



The University of
Nottingham

Characterising Fish Collagen for Expression in Bacterial Systems

Thesis Submitted to the
University of Nottingham for the
degree of Master of Research

Xiaoqin Shi

School of Biosciences
March 2024

Abstract

Isinglass, a collagen-based fining agent commonly used in the brewing industry for centuries, is traditionally produced from the swim bladder of tropical and sub-tropical fishes. However, the production of isinglass raises sustainability concerns, such as overfishing and environmental impact.

Arius maculatus, the species widely used in commercial isinglass production, there is limited data available on the specific protein characteristics of the collagen derived from this source. This study aimed to optimise the extraction and preliminary characterisation of collagen from isinglass from *Arius maculatus* swim bladders in a laboratory setting, addressing the gap in existing knowledge.

The collagen extraction process was refined using acid extraction techniques. The resultants were analysed through SDS-PAGE, revealing the presence of two α -chains and a β -chain with molecular weights slightly over 115kDa and 190kDa, respectively. The study provided essential preliminary data on the molecular structure of this collagen for the future work on the full confirmation of the protein structure. The functionality of these crude extracts as a fining agent was compared against commercial products. The findings reveal that laboratory-extracted protein crude shared structural characteristics with collagen type I and potential in maintaining functionality in finings.

The study also pursued the goal of expressing this collagen in a bacterial host; however, achieving suitable DNA templates for PCR was challenging. Repeated failures with commercial DNA extraction kits led to the development of an optimized protocol using conventional methods, yielding high-molecular-weight genomic DNA (>10 kb) for potential future applications. While time constraints prevented the completion of gene

expression efforts, this research successfully refined both collagen and DNA extraction techniques, providing a critical foundation for future work on sustainable isinglass production and related collagen-based applications.

Keywords: Isinglass; Fish collagen; Heterologous expression; Collagen extraction; DNA extraction

Contents

Abstract	2
Contents	4
List of Abbreviations	6
List of Figures.....	7
List of Tables	9
Chapter 1. Introduction.....	10
1.1 Background and motivation	10
1.2 Isinglass	13
1.2.1 Isinglass as Fining Agent	14
1.2.2 Other Applications of Isinglass	15
1.2.3 Concerns and Limitation of Isinglass	16
1.3 Collagen	18
1.3.1 General Structure and Properties of Collagen	18
1.3.2 Collagen Extraction Methods	21
1.4 Recombinant Techniques and Applications	22
1.4.1 Heterologous Expression	22
1.4.2 Recombinant Collagen and Collagen-like Protein Production...	23
1.5 Bacterial Collagen Like Protein	25
1.5.1 Bacterial Collagen-Like Proteins: Structure and Properties	26
1.5.2 Recombinant Bacterial CLPs	27
1.6 Aim and Objectives.....	28
Chapter 2. Materials and methods.....	29
2.1. Isinglass extraction	29
2.1.1 Buffer Preparation	31
2.1.2 Pretreatment of Fish Swim Bladder	31
2.1.3 Acid Solubilisation and Filtration	31
2.1.4 Isinglass Protein Purification	32
2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.....	33
2.3 Fining Function Test	34

2.4 Genomic DNA Extraction and COL1A2 Gene Isolation	36
2.4.1 DNA Extraction from Fish Swim Bladder Flocs	36
2.4.2 Gel Electrophoresis.....	37
2.4.3 Collagen-Coding Gene Isolation	38
Chapter 3. Results	40
3.1 Collagen Extraction and Analysis.....	40
3.1.1 Results of Collagen extraction	40
3.1.2 SDS-PAGE	45
3.1.3 Fining function test.....	47
3.2 Result of DNA extraction	48
3.2.1 Commercial DNA extraction kits	48
3.2.2 Manual DNA Extraction methods.....	53
3.2.3 Collagen-Coding Gene Isolation	55
Chapter 4. Discussion	59
4.1 Collagen Extraction - Discussion	59
Chapter 5. Conclusion and Future Work	67
5.1 Future Work	67
Acknowledgement	70
Reference List	71

List of Abbreviations

Bcl	Bacillus collagen-like protein
cDNA	Coding DNA
CLPs	Collagen like proteins
dNTP	Deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
gDNA	Genomic DNA
Gly	Glycine
Hyp	Hydroxyproline
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pro	Proline
Scl	Streptococcal collagen-like protein
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TAE	Tris-acetate-ethylenediamine tetra-acetic acid
TE	Tris-ethylenediamine tetra-acetic acid
UV	Ultraviolet

List of Figures

- Figure 1.** Flowchart of key objectives
- Figure 2.** Visualisation of structural characteristics of collagen
- Figure 3.** Whole pretreated catfish swim bladders.
- Figure 4.** Dry fish maw flocs
- Figure 5.** Flow chart showing key steps in Collagen extraction
- Figure 6.** The solidified sample resulted at acid solubilisation step from first attempt of the collagen extraction.
- Figure 7.** Acid solubilised fish swim bladders using updated protocol.
- Figure 8.** Collagen extraction with dry flocs form of fish swim bladders after 2 days of salt precipitation
- Figure 9.** Successful protein precipitation of fish maw flocs in buffer.
- Figure 10.** Collagen sample after 48 hours of freeze-drying.
- Figure 11.** SDS-PAGE of freeze-dried protein samples extracted from whole fish maw and fish maw flocs.
- Figure 12.** SDS-PAGE of freeze-dried protein samples with different ratios of sample loading buffer
- Figure 13.** SDS-PAGE of freeze-dried protein samples under reducing conditions.
- Figure 14.** SDS-PAGE result from the protocol of research article as reference to compare
- Figure 15.** Visualisation result of the first extraction attempt 1 in Table 4. with QIAamp DNA Mini kits.

- Figure 16.** Visualisation result of the extraction attempt with adjustments in Table 5. with QIAamp DNA Mini kits
- Figure 17.** Visualisation result of the extraction attempt in Table 7.
- Figure 18.** Visualisation result of the manual extraction with addition salt precipitation.
- Figure 19.** Gradient PCR result with different annealing temperatures.
- Figure 20.** Gradient PCR result with optimized conditions.

List of Tables

- Table 1.** Fining function test on commercial fining vs. laboratory-extracted collagen.
- Table 2.** Primers used in this study for amplification of collagen-coding genes from fish species
- Table 3.** DNA extraction method using Qiagen DNeasy PowerSoli Kits
- Table 4.** DNA extraction method using QIAamp DNA Mini kits – First attempt
- Table 5.** DNA extraction method using QIAamp DNA Mini kits – Adjustment on the extraction enzyme and sample size
- Table 6.** DNA extraction method using QIAamp DNA Mini kits – adjusted incubation temperature.
- Table 7.** DNA extraction method using Manual extraction

Chapter 1. Introduction

1.1 Background and motivation

Beer is one of the most favoured beverages around the world. In 2019, the average consumption of beer in the United Kingdom was estimated to be 47,119 hectolitres before the pandemic (Conway, 2023). Statistics indicate that the beer industry has recovered from the pandemic's impact, as evidenced by rising figures of annually beer consumption of 83.35 litre per capita in 2021 to forecasted 89 litres per capita in 2024 (Statista, 2024), which in turn has led to increased demand for brewery supplement including fining agents. Among the factors influencing beer quality, clarity is one of the critical determinants of the consumers acceptance and market success (Bamforth, 2018; Mastanjević et al., 2018; Ryder, 2017)

Isinglass, is a traditional collagen-based fining agent with a long history of commercial use, yielding satisfying results. Isinglass is derived from the collagen in the swim bladders of fish from tropical and subtropical waters and has been one of the most reliable and commonly used fining agents in the industry for centuries (Hickman et al., 2000). Other fining agents including gelatine, bentonite, and Polyvinylpolypyrrolidone (PVPP), are also used in the industry, each of them offering distinct advantages. Like isinglass, gelatine is protein-based and effective fining agent but facing the similar sustainability concerns with isinglass (Sunday, 2018). Bentonite is a clay mineral which is popular in wine production but due to its lower protein-binding affinity made it less effective in beer production (Marchal and Waters, 2010). PVPP is a (Cimini and Moresi, 2017)

The basic working mechanism of isinglass as fining agents is based on the nature of the protein molecule that carries a charge which is able to attract the negatively charged haze-forming particles floating in beer, such as the

yeast cells and the insoluble residues, leading to flocculation in the beer. The flocs sediment as the greater particles resulting the higher rate of sedimentation according to the Stokes Law.

In contrast to natural sedimentation, where yeast cells and protein residues in freshly brewed beer may take an extended periods to settle often exceeding the timeframe of the best taste or even the shelf life of the beverage. With the addition of isinglass, the isinglass and yeast/residues complexes sediment faster settling to the bottom of the container and giving a clear and brighter beer product in an ideal timeframe for the best quality beverage. (Leiper, Duszanskyj and Stewart, 2002; Walker, Camarena and Freeman, 2007)

However, recent decade has raised some concerns for using Isinglass including the changes of import and outport policies between countries, environmental concerns, and growing attentions in sustainability globally. Peoples' increasing awareness of marine pollution raises questions on the safety and sustainability of the sourcing from the oceans, for example the release of nuclear water into the ocean increasing the doubts of safety of the marine-sourcing products from the consumers as there are many unknown impacts (Nogrady, 2023).

In response, brewing supplement industries and researchers are exploring sustainable alternatives to traditional Isinglass. This includes finding other fining agents that are both environmentally friendly and cost-effective, as well as utilising advances in biotechnology to replicate the clarifying properties of Isinglass without relying on marine sources (Walker, Camarena and Freeman, 2007).

Rapid advancements in biotechnology in recent years have focused significantly on sustainability as mentioned. Researchers are developing various techniques of cell factories using biotechnological methods, such as

employing plant cells to produce pharmaceutical proteins and utilizing non-pathogenic bacteria as cell factories through genetic manipulation to manufacture heterologous proteins. These cell factories have a wide range of applications, from pharmaceuticals and everyday products to new bioenergy sources (Andersen and Krummen, 2002; Gupta and Shukla, 2015; Peebo and Neubauer, 2018).

The heterologous expression of collagen is of great interest to the industries and researchers. Collagen is one of the most abundant proteins in animals and humans, is a critical component of various organs, tissues, and body structures. Research into collagen, whether for health supplements, medical materials, cosmetics, or food industries, has always been robust and proven. There have been intensive studies on alternatives and heterologous production of collagen, including using plants and bacteria as cell factories to produce medical-grade adhesives and other products. (Avila Rodríguez, Rodríguez Barroso and Sánchez, 2017; Bulleid, John and Kadler, 2000; Olsen, 2003; Yang et al., 2004)

1.2 Isinglass

Isinglass has consistently been a top-choice fining agent in the brewing industry for its cost-effectiveness and straightforward application. The global isinglass market is projected to experience growth, with reports indicating a compound annual growth rate of approximately 4.7% from 2024 to 2030. This growth is driven by increasing demand in the brewing and winemaking sectors, where isinglass is extensively used as a fining agent to clarify and stabilize beverages (USD Analytics, 2024).

The term 'Isinglass' is originated from the Dutch word for sturgeon's swim bladder. The dried form of clean fish swim bladders, often referred to as fish maws (Sarkar et al., 2023), is crucial not only for isinglass production but also as a source of gelatine in food and therapeutic applications, and as a delicacy in Asian countries. There are multifaceted the use of isinglass, with an emphasis on its brewing and biotechnological applications.

Isinglass is predominantly composed of collagen protein around 98% in dry form, that is soluble in organic acids (Hickman et al., 2000). Collagen is the predominant structural protein in the extracellular matrix of various connective tissues, and is the most abundant protein in human and animals, constituting about 25% to 35% of the body's total protein content (Shenoy et al., 2022; Tarnutzer et al., 2023). The unique triple helix structure of collagen, comprises of two $\alpha 1$ chains and a slightly different $\alpha 2$ chain, is essential for isinglass's effectiveness. The amino acid compositions of the collagen molecule differ from the different fish sources. The molecular weights of the collagen molecular weight vary as well depending on the source.

The collagen in isinglass forms a triple helix structure, consisting of amino acids that include a high hydroxyproline content, which is critical for

maintaining its functionality in beverage clarification (Leach, 1967). The hydroxyproline content in collagen from isinglass is influenced by the environmental temperature of the fish which is a crucial factor in determining the denaturation temperature of the collagen molecules. This aspect is essential to understanding of why collagen from isinglass found in fish denature at a lower temperature compared to the bovine collagen. The studies also found that the denaturation of collagen from isinglass is characterized by an irreversible rate process (Hickman et al., 2000; Leach, 1967). There are thermally labile domains within the collagen molecule act as the initiation point of denaturation. Study found that there are 41 residues in the labile region within collagen from isinglass molecule which is attributed to the lower hydroxyproline levels in fish collagen contrasting to the 66 residues within the labile region typically found in mammalian collagen. (Hickman et al., 2000)

1.2.1 Isinglass as Fining Agent

When isinglass is used as fining agent for beverages, the structure and properties of the collagen enable the clarification through distinctive mechanism. The primary function is to form electrostatic complexes with suspended polyphenolic and protein components in the beverages like beer, and with the suspended yeast cell which carries a negative charge in beers (Cosme, Ricardo-da-Silva and Laureano, 2009). These interactions lead to the aggregation of substances then formation of a stable sediment. Then the precipitates settle to the bottom of the container enabling the clear beverage to be taken out without disturbing the sediment. This process is crucial for achieving the desired clarity/appearance and the quality in the final product to the consumers. (Cosme, Ricardo-da-Silva and Laureano, 2009; Hickman et al., 2000; Leiper, Duszanskyj and Stewart, 2002; Walker, Camarena and Freeman, 2007). In summary, the solubility, oligomer

formation, and specific denaturation characteristics of collagen from isinglass are key to its effectiveness in this role.

Commercial isinglass-based fining agents are provided in different forms including, high concentration paste, fine powder, granulated solid, liquid and flocs. The fining agents can be either directly added to the beverage with suggested ratio in the barrels or resuspension/dilutions needed to be performed before applying. The effectiveness of the isinglass fining differs in the form of fining products and the preparation of the finings before adding to the beverage (Murphy and Son, n.d.). Other factors affecting the efficiency of the fining process including the storage conditions of the fining products and the temperature of the beverage to be added. Individual beverage product would have different results in fining using isinglass depending on the yeast strain used for the production of beer and the cell counts in the final product before clarification. Also, isinglass fining agents are very sensitive to the pH of their working environments, thus, the pH of the beverage product and the isinglass fining is crucial to the rate of fining efficacy (Leach, 1967).

1.2.2 Other Applications of Isinglass

Isinglass have been employed not only as fining agents in the brewing industry but also in other research areas and in for different end applications.

Traditionally, isinglass is used for medical uses and as ingredient in paintings in art (Wills, 2020). As a natural biopolymer, isinglass draws the interest of the researchers to develop more novel approaches using isinglass. In 1998, a study had suggested that isinglass adhesive has potential applications for paper conservation which was inspired by the Russian using isinglass and

fish gelatine as traditional adhesives in painting and icon restoration (Petukhova, 1998).

Isinglass is gaining attention in biomedical fields due to its purity and biocompatibility. The low antigenicity of isinglass makes it attractive as an alternative for medical applications including as potential scaffold in tissue engineering (Wang, Wang, & Dong, 2023; Lee et al., 2018), medical adhesive in wound recovery (Sarkar et al., 2023b) and blood plasma substitute (Nature, 1944). Furthermore, the unique physical and chemical properties such as its ability to form strong gels which have implications in both food technology and pharmaceuticals (Lim et al., 2019; Marques et al., 2019). According to Jeżowski and Kowalczewski (2023), the isinglass biopolymer membrane could be an interesting alternative as green energy storage, which would be more environmentally friendly and cost-effective (Jeżowski and Kowalczewski, 2023).

1.2.3 Concerns and Limitation of Isinglass

In the brewing industry, while isinglass effectively clarifies beer and wine, several concerns have been raised by the public and the industry. These concerns primarily including environmental sustainability, ethical considerations, and the impact on product quality.

The primary source of isinglass, the tropical and subtropical fish species, raises questions about the long-term sustainability and safety of isinglass production. Ocean pollutions including microplastics has raised concerns about biological environment in the ocean and food sourcing from the ocean. One of the main habitats of the fish species for isinglass, India ocean, was studied around the microplastics pollution and the toxicity to the raw material (Nugawela et al., 2023). Recent news on the nuclear leak in the United Kingdom (Isaac and Lawson, 2023) and nuclear water release from Japan (Wong, 2023) significantly enhanced public's concerns on food

product from the seawater. Out of these environmental factors, the costs, and efforts to import the fish swim bladders as the raw material for the isinglass production would be increased by the import policy changes and health and safety assurances.

The debate between the legislators and the brewing industry on the necessity of labelling for the beverage fined with protein finings which has the potential to trigger allergy reaction is going. Scientists had performed allergic test in laboratory setting with different protein finings, resulting very low risk of finings trigger the allergy reaction in patients who have respective food allergies (Vassilopoulou et al., 2011). Despite the low risk of fining agent as the allergen, the labelling of isinglass is also an ethical issue considering people who are allergic to fish and consumers who are vegetarian or vegan. The awareness of fish-sourced product in the beverage to the consumers would possibly affect the consumers when they are purchasing the drinks.

The brewing supplement industry and the researchers are investing in the research of solutions to concur the limitations and concerns. Finding and developing an alternative of isinglass is one of the possible solutions. Some examples of research including a pea protein shown good performance in clarification of beers which was suggested could be a vegetarian option as a substitute for isinglass with great potential as a vegan option (Walker, Camarena and Freeman, 2007). Researcher at the University of Nottingham discovered a new possible fining agent in the waste stream from hop processing during the brewing process which could possibly provide a true vegan option as fining agent (Gadon, 2020).

1.3 Collagen

Collagen is the most abundant protein in the animal kingdom which plays a vital role in the structural integrity and biological functions of various tissues in the body. Researchers and industries have been investing significant amounts of research in collagen due to its distinctive structure and vital functions across various areas including daily consumables like food, health supplements and cosmetic products. This review aims to provide comprehensive insight into the characteristics and applications of collagen with emphasis on isinglass.

Collagen has been extensively studied for its abundances in the animal bodies that containing connective tissues and valuable potential in both academic research and commercial purpose (Wang, 2021). The study of collagen dates back to the early 20th century, with significant advancements in understanding the structure and functions over decades (Van Der Rest and Garrone, 1991).

The structure of collagen imparts high tensile strength and is crucial for the integrity of tissues like skin, bones, and cartilage in animals. The classification of collagen is based on the structures and functions which defined the 29 types of collagens into several categories that were identified so far. (Shenoy et al., 2022)

1.3.1 General Structure and Properties of Collagen

Main collagens can be seen in two main categories based on their physical molecular shapes: fibrillar and non-fibrillar collagens. Fibrillar collagens such as type I, II and III are most well-studied and abundant in collagen family, primarily contributing to the mechanical strength and structural integrity of various tissues. They are characterised by long, uninterrupted

triple helix which forming fibrils that give strong and flexible in properties. (Qi Jue Chen et al., 2023)

Type I collagen (Col-I) is the predominant collagen protein in human body, constituting over 90% of the total collagen in whole body (Naomi, Ridzuan and Bahari, 2021), which is normally found in skin, bone, tendons, and connective tissues, providing structural support and tensile strength (Exposito et al., 2010). Col-I in fish usually found the major collagen type in the skin, bones, scales and fish swim bladders (Jafari et al., 2020). The molecular structure of Col-I is featured by its unique helix formation consisting of two $\alpha 1$ chains and one $\alpha 2$ chain. This structure imparts high tensile strength which is crucial for the structural support of the tissues. The amino acid compositions within these chains have a typical repeating pattern of Gly-Pro-Hyp where Gly referring to glycine, Pro representing proline and Hyp for hydroxyproline. This unique amino acid sequence is essential for the stability and function of the helix (Shoulders and Raines, 2009).

The triple helical structure of Col-I is maintained by the interchain hydrogen bonding which is primarily involving the hydroxyproline residues. The stability is crucial for the physical properties of the collagen molecules, such as its resistance to stretching and tensile strength. This unique structure also enables the cross-link formation between the collagen molecules, further enhancing the strength and durability of collagen fibres in tissues (Qi et al., 2022). This structural arrangement is achieved by a complex process of gene expression, post-translational modification like prolyl 4-hydroxylation (Bella and Hulmes, 2017). This is a tightly regulated process and begins in the cell with the transcription of collagen-coding genes and proceeds through a series of intra- and extracellular phases, comprising in the formation of mature collagen fibres (Amirrah et al., 2022; Qi Jue Chen et

al., 2023; Soheila Naderi Gharahgheshlagh et al., 2023). As Col-I, collagen type II also forms strong fibres which normally are smaller in diameter than Col-I, typically found in less presentation in tendon tissues. Collagen type III is also fibrillar collagen which has thinner fibres when compared to Col-I (Buckley et al., 2013).

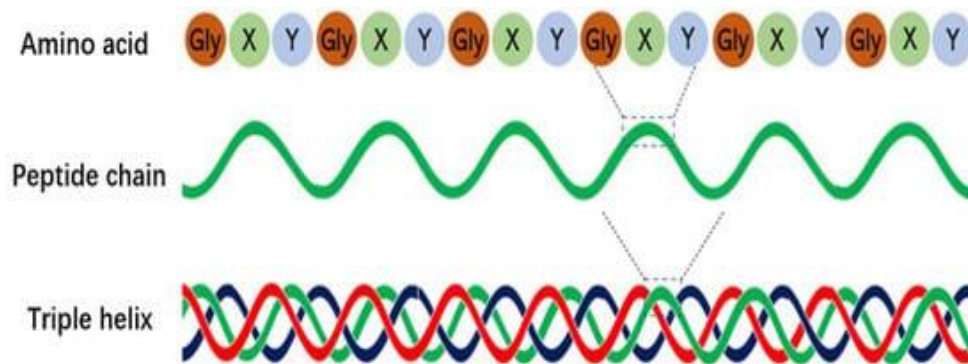


Figure 2. (He et al., 2021). Visualisation of structural characteristics of collagen.

Marine-sourced collagen, particularly from fish, has increasing attention due to their bioavailability and sustainability compared to mammalian sources. In current research on marine-sourced collagen, researchers highlighted that marine-sourced collagen with its beneficial potential in skin health, wound healing, and anti-aging effects (Zdzieblik et al., 2015; Sibilla et al., 2015; De Luca et al., 2016). The unique amino acid composition of marine collagen is instrumental in these beneficial effects, especially the high content of glycine, proline and hydroxyproline (Song et al., 2017; Kim et al., 2018). Additionally, marine-sourced collagen has lower risk of zoonotic disease transmission and allergenicity which are also advantageous for medical and cosmetic applications (Jeevithan et al., 2014; Liu et al., 2015).

1.3.2 Collagen Extraction Methods

The extraction methods of Col-I from source like fish swim bladders necessitates precise techniques to maintain its properties. Acid and enzymatic hydrolysis are two main conventional methods commonly employed in collagen extraction and have been progressively optimised for better yield and purity (Pacak, Powers and Cowan, 2011; Senadheera, Dave and Shahidi, 2020). Recent advancements include the use of new enzymes and mild chemical pretreatments for extraction to preserve the native structure of collagen and enhancing the bioactivity (Ahmad et al., 2017; Bhadra et al., 2021).

A common pretreatment method for collagen extraction involving firstly cleaning the tissue or organ by removal of any dirt or hair. Subsequently, cutting the tissue into small pieces and remove the soluble non-collagenous proteins, polysaccharides and fats from the sample by adding acetate solution and diluted alcohol solution. Then, proceeding onto the extraction, usually acid solubilisation, following with further purification and lyophilisation of the collagen sample. (Wang et al., 2017)

1.4 Recombinant Techniques and Applications

Recombinant protein production is a cornerstone of modern biotechnology, with significant implications across various fields, including healthcare, research, and the food and beverage industries. This technology provides powerful tools for the manipulation of genetic material to produce specific proteins for various applications across different sectors (Wingfield, 2015). Heterologous expression is one of the fundamental methods which enables the production of recombinant proteins in selected foreign host organisms via genetic manipulation and facilitates the synthesis of proteins not naturally present in the hosts (Barnes, Bentley and Dickson, 2003; Rosano and Ceccarelli, 2014). The expression of recombinant proteins involves the transcription of the inserted target gene into to vector and subsequent translation into protein. In the brewing industry, fining agents like isinglass, derived from fish swim bladders, plays a crucial role in clarifying beverages and has been popular and successful for centuries. However, the demand for vegan-friendly, sustainable, and cost-effective alternatives has grown, spotlighting the potential of recombinant proteins as novel fining agents.

1.4.1 Heterologous Expression

Heterologous expression is crucial for producing foreign proteins in host organisms. The choice of expression system is influenced by factors including protein yield, correct protein folding, and specific post-translational modifications (Rai and Padh, 2001). Cloning vectors are DNA molecules used to transport foreign genetic material into a host cell, where it can be replicated and expressed. Plasmids, bacteriophages, and yeast artificial chromosomes are among the most commonly used vectors. The choice of vector is influenced by several factors, including the size of the DNA to be cloned, the need for expression of the cloned gene, and the host cell to be used (Ar et al., 2016; Ongley et al., 2013). This process can be

precisely controlled in the recombinant system to optimise the yield and functionality of the desired protein. This optimisation on the gene expression can be achieved by optimal host-vector system, use of strong promoters and the modification of copy numbers to match the host preferences.

The host system of recombinant protein production varies from bacteria to mammalian cell lines. *Escherichia coli* or *E. coli* has been the favourite bacterial host to the researcher and industries because it is well studied, high competency and relatively ease to manipulate which makes it the first choice as the host system in development of new recombinant proteins. The yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) is another popular host system, that benefits from its characteristics closer to mammalian eukaryotic cells and the ability to perform of post-translation modifications in protein synthesis. (Gomes et al., 2016; Vieira Gomes et al., 2018; Wang et al., 2017)

1.4.2 Recombinant Collagen and Collagen-like Protein Production

As the production of various recombinant protein be engineered to replace their natural counterparts, recombinant collagen requires to be engineered with key physicochemical properties that align with the original collagen molecules found in origin tissues (Parry and Squire, 2017, pp.601–629). The advent of recombinant techniques has allowed the production of collagen and collagen-like proteins in host organisms which offering sustainable alternatives to extraction from natural sources (Fertala, 2020).

However, one of the challenges of recombinant collagen in microbial host systems is the lack of post-translational modifications which is essential for unique triple helical structure of collagen molecules. Researchers have been finding solutions to overcoming this issue by optimising the culture

media with enriched proline and hydroxyproline (Buechter et al., 2003) and engineering the host strains like *E. coli* to be able to produce co-factor which catalyse hydroxylation (Pinkas et al., 2011). The discovery of collagen like protein found in some bacteria species broadened the accessibility and potential of recombinant collagen productions.

Recombinant technology has been employed to produce collagen and collagen-like proteins, overcoming the limitations associated with traditional extraction methods from animal tissues, such as variability in quality and potential for disease transmission. Recombinant protein technology, facilitated by heterologous expression, presents a possible pathway with great potential to developing alternatives to isinglass for beverage clarification utilising the advancement of microbial biotechnology. Despite the challenges, the potential for creating sustainable, allergen-free, and vegan-friendly fining agents is significant. Future research can also include focus on optimising expression systems, ensuring regulatory compliance and consumer acceptance, and assessing the environmental impact of these novel agents.

1.5 Bacterial Collagen Like Protein

This section concentrates on current knowledge of bacterial collagen like proteins (Bacterial CLPs), particularly focus on the protein structural properties, synthesis methods and applications. Also, exploration and discussion of the potential of CLPs as a possible alternative or an improvement to isinglass in brewing. Traditional use of isinglass is as clarifying agent in beer, is facing challenges in long-term period due to sourcing and environmental concerns (Alberghini et al., 2022; Smith et al., 2018; Wu, Zhang and Feng, 2023). Bacterial CLPs could offer a more efficient, sustainable, and potentially vegan-friendly alternatives for the brewing industry and the consumers (Ramshaw et al., 2014).

Collagen as described is a primary structural protein found in animals which is known for its unique triple helix structure and functional versatility. Collagen was once believed to be exclusive to multicellular organisms, collagen-like protein has been identified in bacteria, marking a significant shift in the understandings of collagen protein diversity (Rasmussen et al., 2009). In recent years, scientists had discovered number of fascinating collagen-like protein in the different bacteria species which is gaining increasing interests within scientific area.

The structure of Bacterial collagen-like proteins (CLPs) is central to their growing interest. The CLPs was found with unique amino acid sequence which mirrors the triple helix structure characterised by animal collagen. These finding suggests that CLPs could have valuable potential in medical and industrial applications, such as in the development of new tissue, wound healing and serving as a component in medical devices (Xu et al., 2014; An et al., 2014)

The advancements in genetic engineering have provided a new accessibility for the production and modification of bacterial CLPs which broadening their potential applications in various area. CLPs can be tailored to include functional domains from animal collagens, which significantly enhances the versatility for various applications (Peng et al., 2014; Yu et al., 2014). Furthermore, the introduction of specific mutations in these proteins provides a platform for studying protein structure related diseases like Osteogenesis Imperfecta and Ehlers-Danlos Syndrome (Brodsky and Ramshaw, 2017).

1.5.1 Bacterial Collagen-Like Proteins: Structure and Properties

Bacterial collagen-like proteins (CLPs) have recently emerged as a field of intense study due to their structural and functional similarities with eukaryotic collagens, as well as their unique properties. Bacterial CLPs share the characteristic of the distinctive Gly-Xaa-Yaa repeating amino acids sequence of animal collagens which is crucial in the formation of triple-helical structure.

Bacterial CLPs differ with eukaryotic collagens in their composition and post-translational modifications. Animal collagens are characterised by the post-translational modification of proline residues to hydroxyproline which is critical for stabilising the triple helix through hydrogen bonding. One of the most significant differences is the absences of hydroxyproline in bacterial CLPs which is the key residue for stabilising animal collagen triple helices. The bacteria do not have the enzyme prolyl hydroxylase which is responsible for the modifying proline residues post-translationally at the Yaa position into hydroxyproline in animal collagens (Xu et al., 2010).

Despite the absence of hydroxyproline in bacterial CLPs, there were some pathogenic bacteria discovered with expression of protein that form stable triple-helical structure, and play roles in pathogenicity. For instance, the pneumococcal protein A (PclA) found on the cell surface of *Streptococcus pneumoniae* contributes to the invasion and adhesion into host cells (Paterson et al., 2008) and leading to infections, and the Bcl proteins with triple-helical characteristics found in *Bacillus cereus* and *Bacillus anthracis* possibly playing the role in spore-host interactions with exosporium (Sylvestre, Couture-Tosi and Mock, 2002; Todd et al., 2003). There are also CLPs expressions found in non-pathogenic bacteria species are big interests to the researchers in the field.

1.5.2 Recombinant Bacterial CLPs

With the advancement of recombinant techniques, scientists have been successfully expressed bacterial CLPs Scl protein for production. The most common host system is the *E. coli* BL21 strain with plasmid vectors (Sonal Gahlawat, Nanda and Shreiber, 2023). The recombinant Scl was studied being safe to use as novel collagen-based biomaterials in wound healing and tissue engineering so far (An, Kaplan and Brodsky, 2014; Picker et al., 2022). The low or no risk bacterial CLPs have broadened the field of recombinant collagens as novel materials in medical fields but also the cosmetics (Rodríguez et al., 2017; Peng et al., 2015) and great potential in food industries (Jarita Duasa et al., 2021).

1.6 Aim and Objectives

There have been intensive studies on alternatives and heterologous production of collagen, including using plants and bacteria as cell factories to produce medical-grade adhesives and other products. (Avila Rodríguez, Rodríguez Barroso and Sánchez, 2017; Bulleid, John and Kadler, 2000; Olsen, 2003; Yang et al., 2004)

This research aims to investigate collagen characteristics of isinglass to express the same in bacteria such as *Escherichia coli* as cell factories by recombinant techniques.

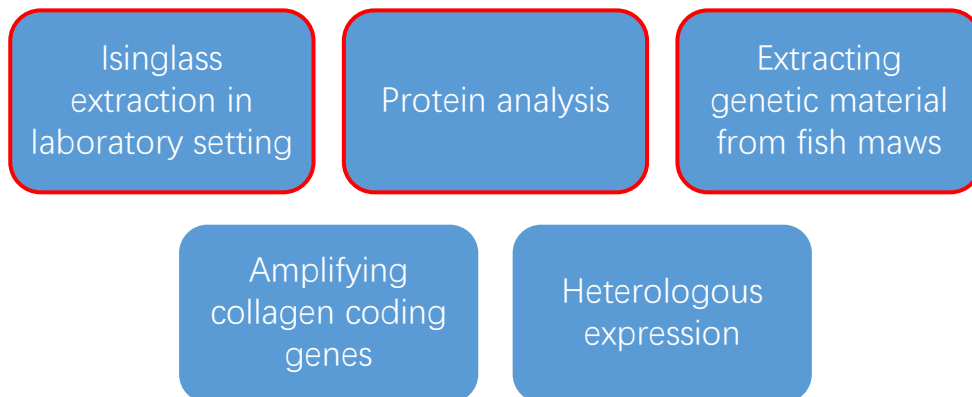


Figure 1. Workflow and key objectives of this study.

Chapter 2. Materials and methods

2.1. Isinglass extraction

The swim bladders samples of *Arius maculatus* were provided by Murphy and Son's Ltd in three forms: pretreated whole fish swim bladders, pretreated dry fish maw flocs, and buffer treated fish maw flocs in storage buffer. Pretreatment was performed by the supplier to remove non-



Figure 3. Whole pretreated catfish swim bladders.



Figure 4. Dry fish maw flocs

collagenous proteins using Murphys's in-house buffer and alkaline solutions. The samples were rinsed under RO water to eliminate the residual impurities after received and before proceeding onto extraction.

The extraction process was carried out under 4 °C and foil covered throughout to prevent solution from evaporating and contamination from the air. This protocol was performed three times with catfish swim bladders in three different forms: buffer washed catfish swim bladder in whole, dry flocs of catfish swim bladders and flocs of catfish swim bladder in storage buffer.

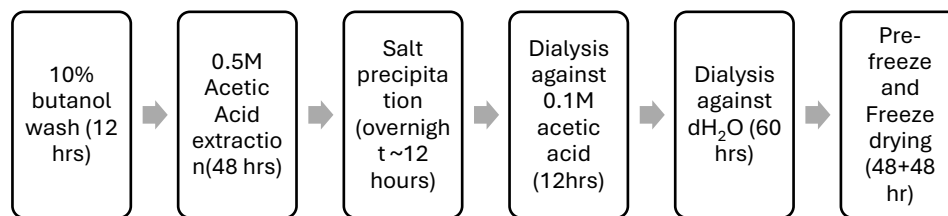


Figure 5. Collagen extraction key steps flowchart.

2.1.1 Buffer Preparation

Two main chemicals used in the collagen extraction, Butanol (99.7%, Sigma-Aldrich) and Acetic acid (99.8 - 100.5 %, Honeywell Fluka™). Butanol was diluted with RO water to the concentration of 10% (v/v) for the defatting treatment. 0.5M acetic acid was prepared with RO water dilution from concentrated to the final concentration of 0.5M for the core step of collagen extraction, acid solubilisation. 0.1M acetic acid was prepared as the dialysis buffer after the acid solubilisation completed.

2.1.2 Pretreatment of Fish Swim Bladder

The first step of collagen extraction from fish is usually alkali solution like NaOH treatment to remove non-collagenous proteins, however in this case, the sample fish swim bladders were pretreated by Murphy and Son's Ltd with in-house alkali solution and Phosphate Buffer Solution (PBS) to remove the non-collagenous proteins.

After the pretreated samples were received, the whole fish swim bladders were initially washed and rinsed under running RO water to remove any residues from the pretreatment, and then the whole fish maw was cut into small pieces around $0.5 \times 0.5 \text{ cm}^3$. The samples were subjected to the defatting step by continuously stirring in 10% butanol with sample/solution ratio of 1:20 (w/v) for 12 hours, solution refreshed around every 3 hours. The samples were then rinse under running RO water and stored in distilled water overnight in cold room at 4 °C.

2.1.3 Acid Solubilisation and Filtration

Butanol-washed samples were then rinsed 3-5 times under running RO water to remove the butanol residues to proceed to the core step of collagen extraction, the acid solubilisation. The washed samples were stirred in 0.5M acetic acid with the sample to solution ratio of 1:50 (w/v)

for 48 hours. The dissolved sample was then filtered with double-layered cheesecloth to remove the insoluble parts.

2.1.4 Isinglass Protein Purification

The filtered sample was then subjected to the salting-out step of extraction by adding NaCl to a final concentration of 2.6M and left at 4 °C overnight to precipitate. The salting-out step is pivotal step in collagen extraction step which utilises high concentration of salts to precipitate proteins from the solution by decreasing the solubility of proteins due to the competition between salt ions and proteins for water molecules leading to protein aggregation and subsequent precipitation. The specific concentration of NaCl created an environment where solubility of the protein is significantly reduced without denaturing its structure to maintain the integrity of collagen's triple helical structure. The process not only enhances the purity of the extracted collagen by removing soluble impurities but also concentrates the collagen in the solution, making it more amenable to further purification and analysis.

The precipitates were collected to proceed on to centrifugation step. The precipitated protein solution was centrifuged at 20000x *g* for 60 minutes at 4°C using Avanti JXN-26 centrifuge, Beckman Coulter. The supernatants were removed after centrifugation and the pellets were retained.

Each of the pellets were resuspended with minimal 0.5M acetic acid then injected to the Slide-A-Lyzer™ Dialysis Kits, 7K MWCO (Thermo Scientific™). After injecting the sample into the dialysis cassettes, process was initiated with 0.1M acetic acid aimed to diffuse out the remained acetic acid in the sample solution for 12 hours with constant stirring at 4°C. Then the sample solution was dialysed against distilled water for 60 hours with constant stirring at 4°C, dialysis water refreshed every 12 hours. It is crucial to

remove the excess salt from the collagen which was achieved through dialysis. This process allowed small molecules like NaCl to diffuse out of the dialysis membrane and retained the larger collagen molecules in the cassettes.

Dialysed protein sample was then transferred to sterile falcon tubes and prepared to be lyophilised or know as freeze-drying by pre-freezing the protein at -20°C overnight and then at -80°C overnight. This pre-freeze step was carried out to shorten the freeze-drying time and retain the structural integrity of the protein. The pre-frozen samples were then sent for freeze-drying for 48 hours. By freeze-drying, the water in the collagen was allowed to be removed without high temperature which can denature the proteins and disrupt the structure (Deyl, Mikšík and Eckhardt, 2003; Santos et al., 2013). Additionally, lyophilised collagen has higher solubility, minimised risks of microbial contamination, for easier and longer storage.

2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used analytical method in laboratories to separate proteins according to their molecular mass (Smith, 1984). The methodology of SDS-PAGE was employed with modifications from the method of Laemmli, 1970.

SDS-PAGE was carried out using precast gel, Bolt™ 4-12% Bis-Tris Plus Gel, and Bolt™ MES SDS Running Buffer both from Invitrogen™. The lyophilised collagen sample were dissolved with distilled water or 0.1M acetic acid for comparisons with the sample and solution of 1mg:1mL (w/v). Bolt™ LDS Sample buffer (4X) was then added to the dissolved sample with desired buffer: sample ratio and followed with heat treatment to fully denature the collagen into linear chain, also facilitate the SDS binding uniformly to the length of the proteins. Heat-treated samples and the protein ladder,

PageRuler™ Plus Prestained Protein Ladder, ranging from 10 to 250 kDa, were loaded on the precast gel, electrophoresis was running with 200V for 30-50 minutes.

The SDS-PAGE of the lyophilised collagen sample was performed with several comparison sets: high and low concentrations of sample, water vs. acid rehydrated sample, different forms of raw material, and reducing vs. non-reducing running conditions. The primary procedures of the method were the same for these comparisons. For the reducing condition, Bolt™ Sample Reducing Agent (10X) was added in a ratio reducing agent: loading sample mixture ratio of 1:10 (v/v).

The gel was stained with Coomassie Blue solution (0.05% Coomassie Blue, 10% glacial acetic acid, 25% isopropanol and 64.5% water) overnight on agitation plate and then destained with destaining solution which was prepared with 10% glacial acetic acid, 30% methanol and 60% RO water overnight, by occasionally refreshing the solution. The gels were then rinsed under running RO water after the destaining was completed for observation. The Coomassie Blue dye binds to the proteins and results in colour change which allows the visualisation of the protein after the destaining process remove exceeded dyes on the gel.

2.3 Fining Function Test

To verify if the collagen extracted under laboratory setting contained the fining functions in beer as commercial isinglass product would have, a simple functional test was carried out. This fining function test method was adopted from the commercial isinglass optimisation procedure to find out the most optimised working concentration to fine the beer or wine, which a common service normally can be provided by the brewery supplement company to the brewers purchased their fining products.

Freshly brewed beer and commercial isinglass product as reference, Finest Triple, which is a concentrated isinglass fining solution were provided by Murphy & Son Limited specifically clarifying the yeast cells in beer.

The lyophilised collagen was dissolved with distilled water to the concentration of 1mg/mL of collagen. Finest Triple was diluted with two portions of distilled water following the manufacturer's guidance as the product was in concentrated form. Diluted commercial isinglass and dissolved laboratory collagen from isinglass were added to 50mL of freshly brewed beer in clear sterilised glass flasks to the desired final concentrations for fining test stated in Table 1., mixed well by gentle inverting the bottles. A blank was created as well by adding nothing to 50mL of the beer. The flocculation can be visible in several hours, and the visible sedimentation can be observed after 24-48 hours.

Table 1. Fining function test on commercial fining aid vs. laboratory-extracted collagen.

Sample	Label as	Amount of sample added (μ L) in 50mL of beer	Final concentration of fining aid in 100ml of beer
Blank	0	0	0
Finest Triple	I1	173.5	0.347
Finest Triple	I2	347.0	0.694
Finest Triple	I3	521.0	1.042
Finest Triple	I4	694.5	1.389
Freeze dried sample	C1	173.5	0.347
Freeze dried sample	C2	347.0	0.694
Freeze dried sample	C3	521.0	1.042
Freeze dried sample	C4	694.5	1.389

2.4 Genomic DNA Extraction and COL1A2 Gene Isolation

In contemporary literature and past studies, there has been an exploration into extracting genes that code for collagen from the swim bladders of diverse fish species (Lin et al., 2020; Lin et al., 2022; Rong et al., 2022). However, the specific species under investigation in this project has not been studied. Identifying an effective method for gene extraction from the particular species in this study, *Arius maculatus*, serving as a foundational step towards the future heterologous expression of the collagen.

2.4.1 DNA Extraction from Fish Swim Bladder Floccs

To identify the most appropriate DNA extraction methodology for this specific species, *Arius maculatus*, both DNA extraction approaches such as commercial DNA extraction kits, Qiagen DNeasy PowerSoil Kits and QIAamp DNA Mini kits, and traditional manual DNA extraction techniques were trialled and tested. The experimentation varied in terms of the swim bladder's forms, dry floccs or those preserved in storage buffer and the quantity of material used (ranging from 10mg to 25mg), seeking the optimal extraction process with both commercial kits and conventional extraction methods.

With the commercial kits, the swim bladder sample was incubated with lysis buffer and the Proteinase K for an hour at 56°C (QIAamp DNA Mini kits) to 60°C (Qiagen DNeasy PowerSoil Kits), following with vortexing to break down the cells thoroughly. For the QIAamp DNA Mini kits, another lysis buffer required to be added after the first-round incubation, and proceed a second round of incubation at 70°C for 10 minutes. After lysis incubation, the samples were transferred into spin columns centrifuged at 6000 g – 10000 g with by Fresco™ 17 Microcentrifuge (Thermo Scientific™) and kits

buffers for salt precipitation, ethanol wash steps. The DNA remained within the membrane of spin column then be eluted with elution buffer after 1 minute incubation at room temperature and centrifuge to sterile Eppendorf tubes.

DNA extraction was performed using a manual protocol for extracting high-molecular-weight DNA from swim bladder tissue. Sample (25mg) were incubated with 0.1M PBS or TE buffer and 20 μ L lysozyme (100mg/mL) at 37°C for 30 minutes. Mechanical disruption was performed with fine glass beads and MagNA Lyser (Roche) to break down the cells. Following centrifugation at 16000 g by Fresco™ 17 Microcentrifuge (Thermo Scientific™) for 10 minutes, the supernatant containing DNA was transferred to sterile tubes.

To purify DNA, ethanol was added to precipitate contaminants, followed by repeated washes with 70% ethanol. The pellet was air-dried and resuspended in elution buffer.

2.4.2 Gel Electrophoresis

To assess the integrity and approximate size of the extracted DNA, gel electrophoresis was conducted. This analysis utilised agarose gel with different concentration of agarose including 1.0% and 1.5%, prepared by dissolving agarose in 1X TAE buffer and heating until fully dissolved. Upon cooling to approximately 50°C, ethidium bromide was added for DNA visualisation under UV light. The gel was cast with a well comb in place and allowed to solidify.

During the cooling phase of the gel, DNA samples were prepared for electrophoresis by mixing with the DNA Gel Loading Dye 6X (Thermo Scientific™) at a volumetric ratio of 1:5 (dye:DNA sample). Two types of

DNA ladders were employed in the experiments: HyperLadder™ 1kb (Meridian) ranging from 200bp to 10Kb and GeneRuler High Range DNA Ladder (Thermo Scientific™) ranging from 10Kb to 48Kb, aimed at determining the optimal DNA ladder to estimate the unknown size of the DNA samples.

DNA samples, alongside DNA ladder were loaded into the wells of the gel. Electrophoresis was run at 100V for 30-45 minutes, ensuring the DNA migrated at a rate allowing for clear separation. Upon completion, the gel was visualized under UV light, and images were captured using GelDoc.

2.4.3 Collagen-Coding Gene Isolation

The polymerase chain reaction (PCR) methodology was employed to amplify specific collagen-coding genes from the DNA obtained from fish swim bladders. The aim was to isolate and amplify the *COL1A2* gene from catfish swim bladders, with the ultimate goal of heterologous expression in bacteria. The PCR running conditions was initially adapted from a study on molecular cloning of type I collagen cDNA in grass carp, and three sets of primers (Table 2.) from Yu et al., 2014 as the initial trial primers in this case. The primers were ordered from Sigma-Aldrich® and the PCR reaction was performed with QIAGEN© Taq PCR Core Kit.

The extracted gDNA of catfish swim bladders was prepared for PCR on ice with a single reaction mix with total volume of 25 µL containing 2.5 µL of 10x PCR buffer, 0.2 mmol of dNTPs, 1 unit of Taq DNA polymerase, 1 µL of gDNA, and 0.2 mmol of each primer. The thermal cycler was programmed for an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 49°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Following PCR, the amplification products

were evaluated through agarose gel electrophoresis to confirm the specificity and efficiency of the amplification.

Table 2. Primers adapted from Yu et al., 2014, for amplification of collagen-coding genes originated from studied fish species

Primer Name	Sequence (5' to 3')	Origin Fish Species	Primer Usage
<i>COL1A2F1</i>	GCTTTGTGGATACCCGGATT	Zebrafish	Core sequence of <i>COL1A2</i>
<i>COL1A2R1</i>	CCAGCGTCACCCTTTCCAC	Zebrafish	Core sequence of <i>COL1A2</i>
<i>COL1A2F2</i>	GGTGAGACTGGCTCCTTTTG	Rainbow Trout	Core sequence of <i>COL1A2</i>
<i>COL1A2R2</i>	CCATGTAGGCGATGCTGT	Rainbow Trout	Core sequence of <i>COL1A2</i>
<i>COL1A2F3</i>	GCCAAGGATTATGAGGTG	Chum Salmon	Core sequence of <i>COL1A2</i>
<i>COL1A2R3</i>	GAGGATGGGAAGGCGAGATG	Chum Salmon	Core sequence of <i>COL1A2</i>

To optimising the PCR conditions, the gradient annealing temperatures was programmed and perform using the ³Prime Techne thermocycler. Gradient PCR was performed to optimise annealing temperatures for primer sets targeting the *COL1A2* gene. Reactions were prepared in a 25 µL volume containing 1 µL template DNA, 1 µL of each primer, 0.2 mM dNTPs, and 1 unit of *Taq* DNA polymerase (Thermo Fisher Scientific™). The gradient ranged from 55°C to 60°C.

Chapter 3. Results

3.1 Collagen Extraction and Analysis

3.1.1 Results of Collagen extraction

The initiate collagen extraction protocol was adopted from studies on collagen extraction of different byproduct of various species of fish. The pretreated whole fish swim bladder (Figure 3.) was used for extraction following the first version protocol. Without the pepsin and its nature of low working temperature as key consideration of the protocol, there was no low temperature restriction throughout the extraction process and foil cover of the container, which led to the solubilised sample significantly thickened to almost solidified jelly-like texture, and the volume of the mixture reduced from 500mL of acid added to around 130mL solution due

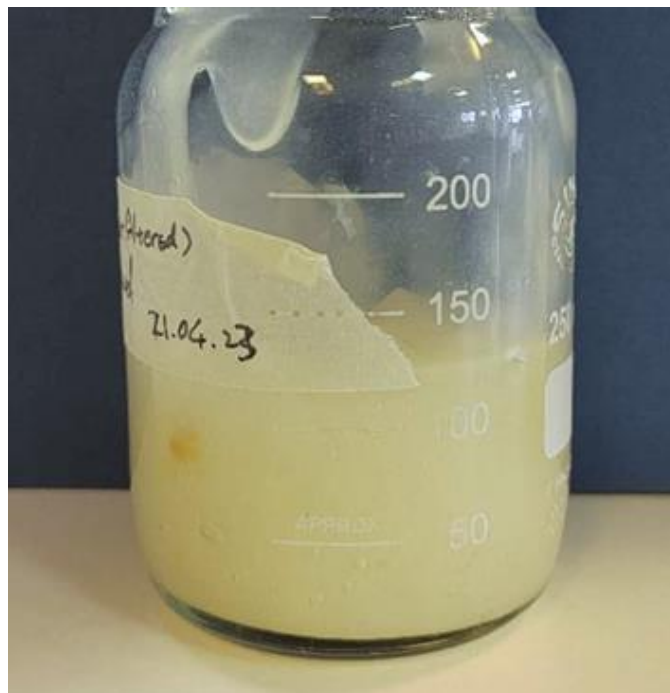


Figure 6. The solidified extraction solution resulted from acid solubilisation step from first attempt of the collagen extraction.

to evaporation (Figure 6.). The solidified solution could not proceed to the filtration thus was disposed.

The procedure was adjusted following an extensive review of literature on collagen extraction methods, especially from fish swim bladders. The revised protocol, inspired by a study on collagen extraction from the skin and swim bladder of seabass by Sinthusamran, Benjakul, and Kishimura (2013). Modifications to the protocol, including the use of foil covers and lower working temperatures, successfully prevented evaporation and improved the solubilisation process.

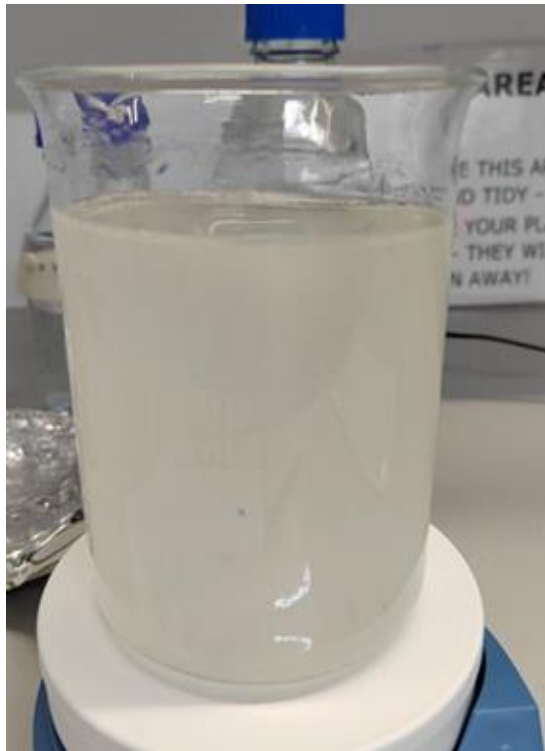


Figure 7. Completed acid solubilised fish swim bladders using updated protocol.

The updated acid solubilisation protocol yielded a clear, viscous solution (Figure 7). Freeze-drying produced white, sponge-like collagen samples (Figure 10), suitable for downstream analysis.

In the first attempt with the updated protocol was the pretreated whole catfish fish swim bladder as shown in Figure 3. The acid solubilised sample in this attempt resulted with a much less viscous solution in Figure 7. compared to the resultant following previous protocol in Figure 6., and there was no loss of the solution due to the prevention of evaporation. The colour of solution was much lighter and whiter colour compared to the one from old protocol.

The second attempt of collagen extraction with the same weight of the sample but in the form of dry fish swim bladder flocs failed at the step of salt precipitation. The solution after completed acid solubilisation was more viscous but not solidifying compared to the solution with whole fish swim bladders. After overnight settlement for salt precipitation, soft clumps which possibly be the protein aggregates was found in the sample solution but there was no visible separation as in Figure 8. The sample was left for addition two days for the precipitation, however no separation of the protein precipitated occurred. The failure of salting-out precipitation led to



Figure 8. Collagen extraction with dry flocs form of fish swim bladders after 2 days salt precipitation of the collagen.

termination of proceeding to collection of supernatants and further steps for this attempt of extraction.

The last attempt of collagen extraction was conducted with fish swim bladder flocs in storage buffer or refer as wet flocs in the following. Same sample size was carried out in this extraction and the salt precipitation was successful with clear separation as shown in Figure 9. which proceeded to further purification steps without complications.

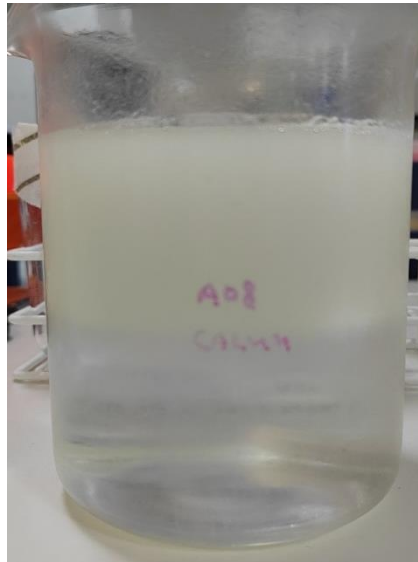


Figure 9. Successful protein precipitation. Sample form: Fish maw flocs in buffer.



Figure 10. Collagen sample after 48 hours of freeze-drying.

Benchtop centrifuge device with 4000 xg as maximum speed was used for centrifugation of samples resulted in loose pellets and visible precipitates floating in the supernatant. Additional time was employed in the centrifugation with this device and extra care was taken when removing the supernatant of the resultant. High-speed centrifuge was used with the speed of 20000 xg for high-speed centrifugation which gave firmer pellet and no visible precipitation in the supernatant. As in result, higher speed of the centrifugation did significantly reduce the time for centrifugation and sample loss compared to the lower speed centrifugation of the sample.

The dialysed sample was clear with gel like texture that was subjected to freeze-drying. The lyophilised collagen samples resulted with white in colour and sponge-like texture which had flexibility and unable to be smashed or grinded into powder forms as other freeze-dried substances. Additional 48 hours of freeze-drying was tried to determine whether there is any change in weight or texture on the samples which resulted in no difference at all. Both 48 hours freeze-dried sample and 72 hours freeze-dried ones gave dry sponge-like texture without weight changes which mean the 48 hours of lyophilisation removed the moist completely, and texture was not caused by the exceed moist in the sample. Every 1mL of the dialysed sample before freeze-drying resulted approximately 1mg of lyophilised collagen.

The solubilities in acid and distilled water of the freeze-dried collagen samples in this case were compared as well. The freeze-dried collagen dissolved faster in the 0.5M acetic acid compared to the one dissolved in distilled water with the same final concentration of the collagen. The SDS-PAGE analysis had shown no difference of the collagen dissolved in acid and distilled water.

3.1.2 SDS-PAGE

Two clear protein bands could be observed in all SDS-PAGE results sized around molecular weights slightly over 115kDa and over 190kDa (Figure 11.). Compared to the past studies on collagen from isinglass, this pattern was showing the α chains and the β -chain of the collagen. The collagen samples ran under reducing condition SDS-PAGE had resulted no difference to the non-reducing ones which indicated no presence of disulphide bonds in the molecule of this particular collagen samples (Figure 13).

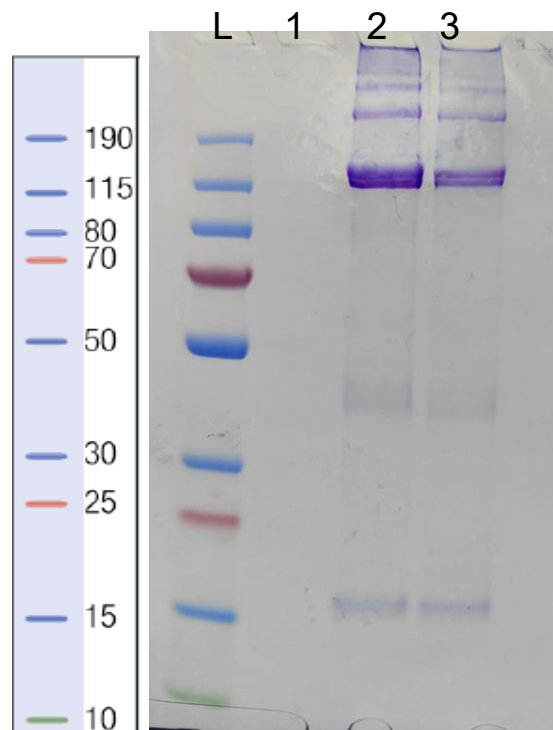


Figure 11. SDS-PAGE of freeze-dried protein samples extracted from whole fish maw and fish maw flocs. L- Protein Marker (ladder); 1 – negative control (water); 2 – rehydrated collagen from whole swim bladder; 3 – rehydrated collagen from wet swim bladder flocs.

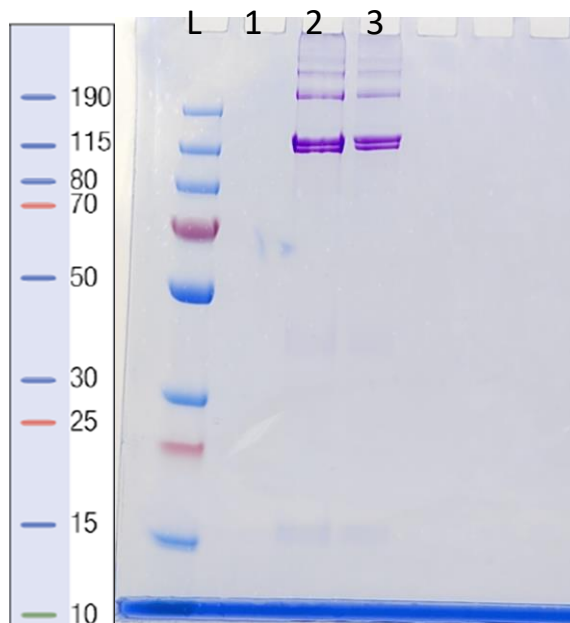


Figure 12. SDS-PAGE of freeze-dried protein samples with different ratio of loading sample buffer: collagen sample. 2 – rehydrated collagen: Sample buffer (1:1); 3 – rehydrated collagen: dH₂O : sample buffer (2.5: 6.5: 1).

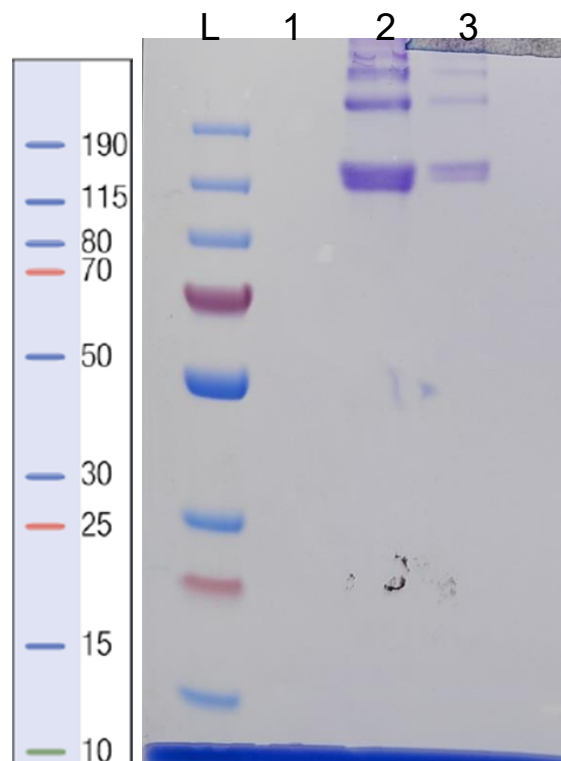


Figure 13. SDS-PAGE of freeze-dried protein samples under reducing conditions. L- Protein Marker (ladder); 1 – negative control (water); 2 – rehydrated collagen from whole swim bladder; 3 – rehydrated collagen from wet swim bladder flocs.

Interestingly, an SDS-PAGE was conducted with laboratory extracted collagen samples had almost the same result with diluted commercial isinglass fining, Finest Triple. The commercial isinglass had resulted slightly less sharp bands on the gel compared to the laboratory extracted ones but the visible two α chains and the β -chain pattern. This means either the collagen concentration and purity are high in commercial product, or the laboratory extracted collagen need to be purified further with alternative protein purification methods. Methods like western blotting to verify the desired collagen in the sample or commercial products can be conducted for the confirmation in the future.

3.1.3 Fining function test

To assess the functionality of the laboratory-extracted collagen from isinglass as a fining agent in beer, a simple comparative fining test with commercial isinglass was conducted. The commercial isinglass fining, diluted with two portions of distilled water, was added to freshly brewed beer as per the product's instructions. The collagen sample was dissolved in distilled water but not acetic acid, to ensure the pH did not fall below the operational pH range of fining agents, achieving a final concentration of 1mg/mL. The beer samples with addition of finings and collagen both showed visible flocculation and sedimentation after overnight time. While the commercial isinglass fining had the best sedimentation result with third test concentration, the lyophilised sample gave the clearest beer under observation with the second test concentration which was lower. The results had shown that the laboratory extracted collagen from fish maw remain the fining functions as the commercial isinglass fining agents.

3.2 Result of DNA extraction

DNA extraction aimed to result high-quality genetic material for PCR amplification of collagen-coding genes. The commercial DNA extraction kits adapted from formal researches yielded likely be degraded DNA (Figure 14.). Manual extraction protocols employing salt precipitation improved the DNA extract quality and giving clear high-molecular weight DNA result as shown in Figure 17 which suitable for future analysis and downstream PCR.

3.2.1 Commercial DNA extraction kits

Qiagen DNeasy PowerSoil Kits was the first kit tried for DNA extraction from the fish maw dry flocs due to it is featuring as a powerful tool in DNA extraction with various types of the biological samples to soil samples. By following the manufacturers' instruction of DNA extraction with tissue samples, 3 attempts with this extraction kits were performed with a series of adjustments on the samples size from 25mg to 200mg and the lysis enzyme as shown in Table 3.

Table 3. DNA extraction method used: Qiagen DNeasy PowerSoil Kits

Attempt	Sample	Sample amount (mg)	Enzyme
1	Catfish Dry Flocs	200	Proteinase K
1	Catfish Dry Flocs	200	Proteinase K
2	Catfish Dry Flocs	50	Proteinase K +20 μ L of 100mg/mL lysozyme
2	Catfish Dry Flocs	100	Proteinase K+20 μ L of 100mg/mL lysozyme
3	Catfish Dry Flocs	25	Proteinase K
3	Catfish Dry Flocs	25	Proteinase K+20 μ L of 100mg/mL lysozyme

The first attempt encountered difficulties on fully complete tissue digestion of the samples due to the large sample size of 200 mg of dry flocs yielded

no observable outcomes on the visualized agarose gel. Consequently, the sample size was reduced to 100mg and 50mg, and addition lysozyme was incorporated in the subsequent trial with the identical extraction kit. Nonetheless, the following gel electrophoresis resulted no visible result of DNA. Third attempt was performed with further reduced sample size and two different enzymatic conditions for incubation, however, no DNA result detected.

Reconsideration of the commercial DNA extraction kits choice was taken after the failures from the result. QIAamp DNA Mini kits (Zhang et al., 2022) was chosen after further literature research on DNA extraction methods from fish swim bladders. The first attempt (Table 4.) using this kit was carried out with four different sample sizes, and the extract crude was examined with nanodrop to determine the DNA concentrations. The initiate DNA ladder employed was the GeneRuler High Range DNA Ladder from Thermo Scientific™, ranging from 10171 to 48502 bp.

Table 4. DNA extraction method used : QIAamp DNA Mini kits – First attempt

Sample	Sample amount (mg)	Labelled as	Labelled on gel	Note
Catfish Dry Flocs	10	10mg	10	Nanodrop ng/ μ L=8.4
Catfish Dry Flocs	15	15mg	15	Nanodrop ng/ μ L=14.8
Catfish Dry Flocs	20	20mg	20	Nanodrop ng/ μ L=16.5
Catfish Dry Flocs	25	25mg	25	Nanodrop ng/ μ L=23.0

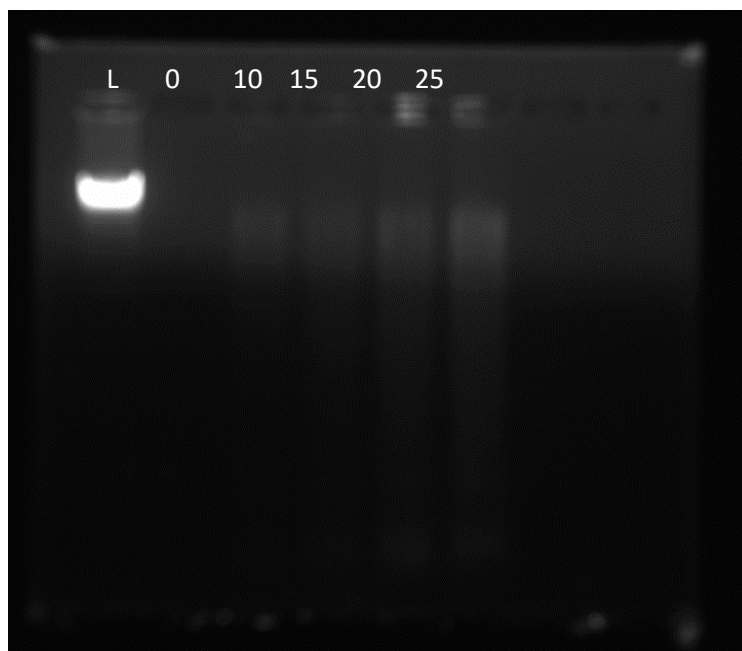


Figure 14. Visualisation result of the extraction attempt 1 in Table 4. with QIAamp DNA Mini kits. Gel electrophoresis ran with 1.5% agarose gel at 65V for 60 minutes. L- GeneRuler High Range DNA Ladder; 0- water + loading dye.

As the visualisation shown in Figure 14, the DNA seemed degraded with all extract crude from different sample sizes, and there was no visible separation of the DNA ladder. The nanodrop result in Table 4. might have detected the degraded DNA or DNA-like contents in the crudes.

The adjustments on sample size and the enzymes used in extraction was made and the extraction with adjustment were attempted as recorded in Table 5. Alongside further adjustments of the gel electrophoresis conditions included: adjusting loading concentration of the high range DNA ladder by dilutions commercial ladder with water tempted to achieve visible separation to provide reference for sample DNA size. The concentration of agarose gel was trailed from 0.8% to 1.5% to determine the ideal matrix for this DNA ladder separations and the detection of the DNA samples in Figure 15. Different electrophoresis running voltage and duration lengths were applied corresponding agarose gel concentration respectively.

Unfortunately, the results of these attempts remained the same as in Figure 14, the nanodrop results were similar to the ones in Table 4. HyperLadder™ 1kb (Meridian) was employed in gel electrophoresis as well to examine the

Table 5. DNA extraction method used : QIAamp DNA Mini kits – Adjustment on the extraction enzyme and sample size

Sample	Sample amount (mg)	Labelled as	Note
Catfish Dry Floccs	20	20mg	Proteinase K
Catfish Dry Floccs	25	25mg	Proteinase K
Catfish Dry Floccs	30	30 P	Proteinase K as lysis agent
Catfish Dry Floccs	30	30 L	20µL of 20mg/mL lysozyme as lysis agent instead of Proteinase K came with the kit
Catfish Dry Floccs	25	25 L+P	Both 20µL of Proteinase K and 20µL of lysozyme stock were added as lysis agent

DNA size and verifying the electrophoresis functioning properly as shown in Figure 15.

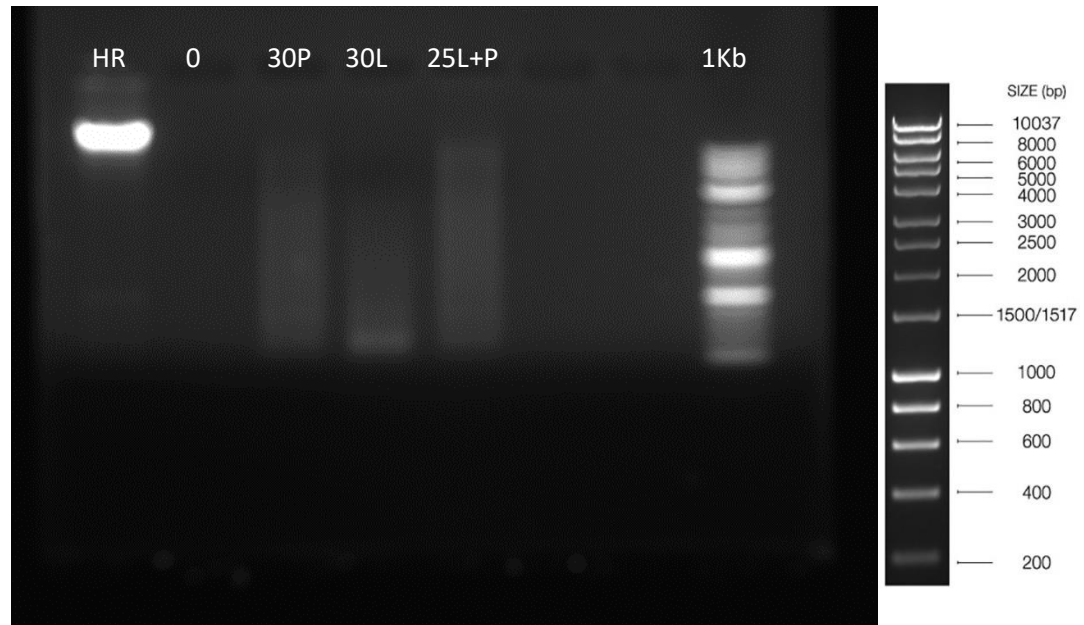


Figure 15. Visualisation result of the extraction attempt in Table 5. with QIAamp DNA Mini kits. Gel electrophoresis ran with 1.5% agarose gel at 100V for 30 minutes. HR- GeneRuler High Range DNA Ladder; 0- water + loading dye; 1Kb- HyperLadder™ 1kb (Meridian)

To examine the cause of DNA degradation, adjustments on the incubation time and temperature of the DNA extraction was made and tested as recorded in Table 6. The incubation time was reduced from 90 minutes as suggested in the manufacturer's instruction to 60 minutes. one of the samples was incubated with lysozyme at 37°C for lysozyme activation,

Table 6. DNA extraction method used : QIAamp DNA Mini kits – adjusted incubation temperature.

Sample	Sample amount (mg)	Labelled as	Nanodrop ng/μL	Note
Catfish Dry flocs	25	37	32.5	incubation temperature at 37°C for 1 hour
Catfish Dry flocs	25	56	33.0	incubation temperature at 56°C for 1 hour

where another was incubated with Proteinase K at 56°C for enzyme activation. The reduced temperature with lysozyme-sample and incubation time was applied to detect whether the DNA degradation was caused by the over incubation. The result of gel electrophoresis indicated DNA degradation again even the DNA concentration detected by the nanodrop increased compared to the previous ones.

3.2.2 Manual DNA Extraction methods

The optimised DNA extraction method or referred as the manual DNA extraction was employed after the extraction using commercial kits failed even with a series of adjustments. The first attempt with manual methods was only performed with dry swim bladder flocs sample, the DNA crude was more viscous compared to the ones extract crude using the commercial kits. The written record on this attempt stated there was visible fluorescence under observation via UV illustrator around the sample loading well. The extract crude was diluted with additional elution buffer and gel electrophoresis was performed again, resulted nothing on the gel.

Table 7 recorded the second attempt of manual extraction with both dry flocs and wet flocs. D and W represent the form of the extraction sample, D represent dry flocs and W represent the wet flocs; P and T indicate the incubation buffer used for the extract where P stands for PBS solution and TE buffer. The result in as shown in Figure 16. displayed the visible fluorescence around the wells where the DNA crude extracted from the dry flocs with both incubation solution of PBS and TE. The DNA concentration of the DP and DT sample in Table 7. were measured with remaining samples

using the nanodrop and the results was 28.5 ng/μL and 18.5 ng/μL respectively.

Table 7. DNA extraction method used : Manual extraction

Sample	Sample amount (mg)	Labelled on gel	Note
Catfish Dry Flocs	25	DP	DNA manual extraction using PBS
Catfish Dry Flocs	25	DT	DNA manual extraction using TE buffer
Catfish Flocs in buffer	25	WP	DNA manual extraction using PBS
Catfish Flocs in buffer	25	WT	DNA manual extraction using TE buffer

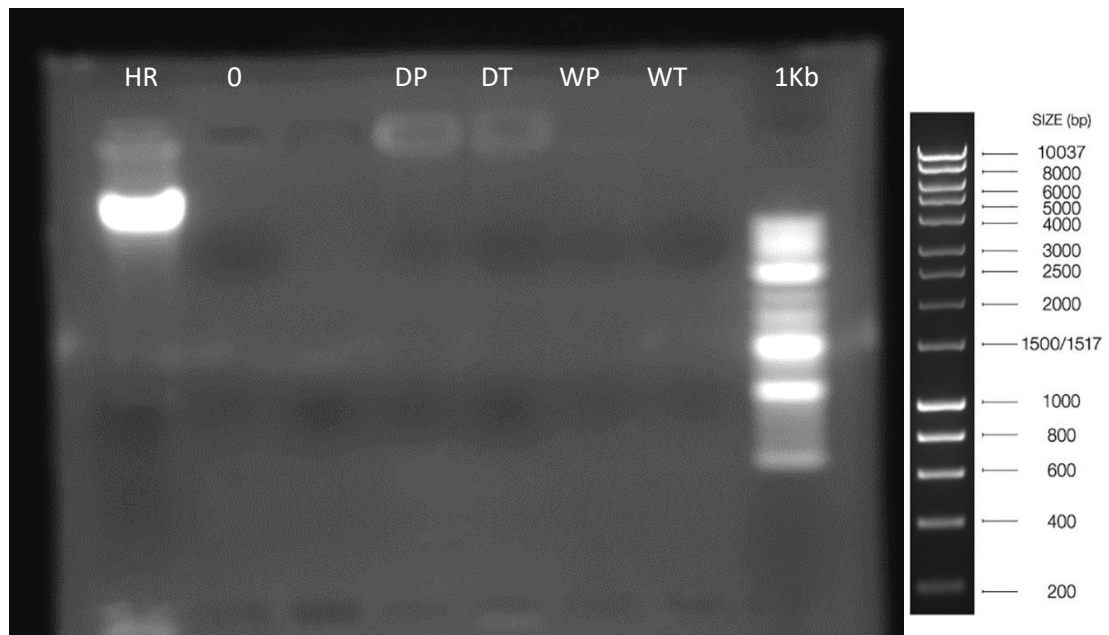


Figure 16. Visualisation result of the extraction attempt in Table 7. with manual extraction methods. Gel electrophoresis ran with 1.5% agarose gel at 100V for 30 minutes. HR- GeneRuler High Range DNA Ladder; O- water + loading dye; 1Kb- HyperLadder™ 1kb (Meridian)

Two more attempts on the manual extraction method with the dry flocs was carried out with changes with the elution buffers and ran with different concentration of the agarose gel also the voltage and time. Resulted no visible bands nor even fluorescence around the wells under visualisations.

By adding high-concentration salt composed by NaCl and distilled water to the final concentration of 6M salt solution before ethanol precipitation, the cell debris and the proteins can be precipitated and removed by centrifugation. The optimised procedure was performed with both dry and wet flocs. The result had shown clear sharp DNA band with the crude extracted with wet flocs under UV illuminator. According to the DNA ladder, DNA sized greater than 10Kb as indicated in Figure 17.

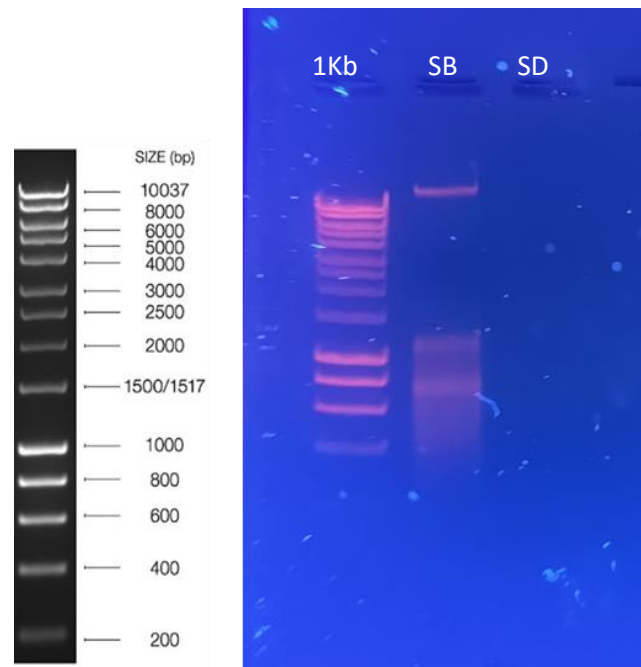


Figure 17. Visualisation result of the manual extraction with addition salt precipitation. Gel electrophoresis ran with 1.5% agarose gel at 100V for 30 minutes. 1Kb- HyperLadder™ 1kb (Meridian), SB- DNA extracted from wet flocs; SD – DNA extracted from dry flocs.

3.2.3 Collagen-Coding Gene Isolation

As one of core composition of collagen molecules, the α chain coding gene was selected as the initiate target in collagen-coding gene isolation. The PCR methods and the primer sequences were adapted from Yu et al., 2014.

Following the methods and PCR condition from the original paper, PCR was performed with DNA extracted by manual extraction as the template DNA, there was no resultant gene detected. Thus, optimisations on the PCR were carried out against both the annealing temperature and slight modification on the master mix ratios.

Out of all attempts of PCRs, the primers set of *COL1A2F1* and *COLA2R1* which originated from zebrafish showed no resultant gene at all through. After adjusting the annealing temperature, the other two pairs of primer originated from rainbow trout and chum salmon produced results with multiple bands on the visualised gel as indicated in Figure 18 and 19, which were difficult to identify the target gene fragment. The result in Figure 18 and 19 indicated some amplification of gene fragment with the primer pairs *F2R2* and *F3R3* originated from rainbow trout and chum salmon. The increased annealing temperature in Figure 19 resulted reduced gene

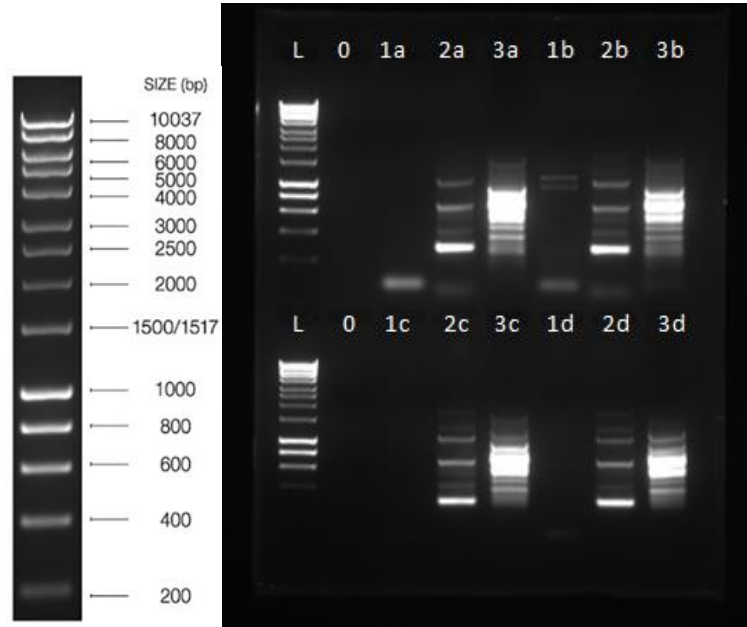


Figure 18. PCR resultant with gradient annealing temperatures. Letters indicate the temperature: a – 50°C, b – 51.1°C, c – 53.5°C and d – 54.7°C, number indicate the primer pair. 1 – *COL1A2F1* and *COL1A2R1*, 2- *COL1A2F2* and *COL1A2R2*, 3- *COL1A2F3* and *COL1A2R3*. Negative control indicated as 0. Running conditions: 1.5% agarose, 95V, 40 minutes.

amplifications compare to result indicated in Figure 18 with lower annealing temperature. These results provided a narrowed ideal annealing

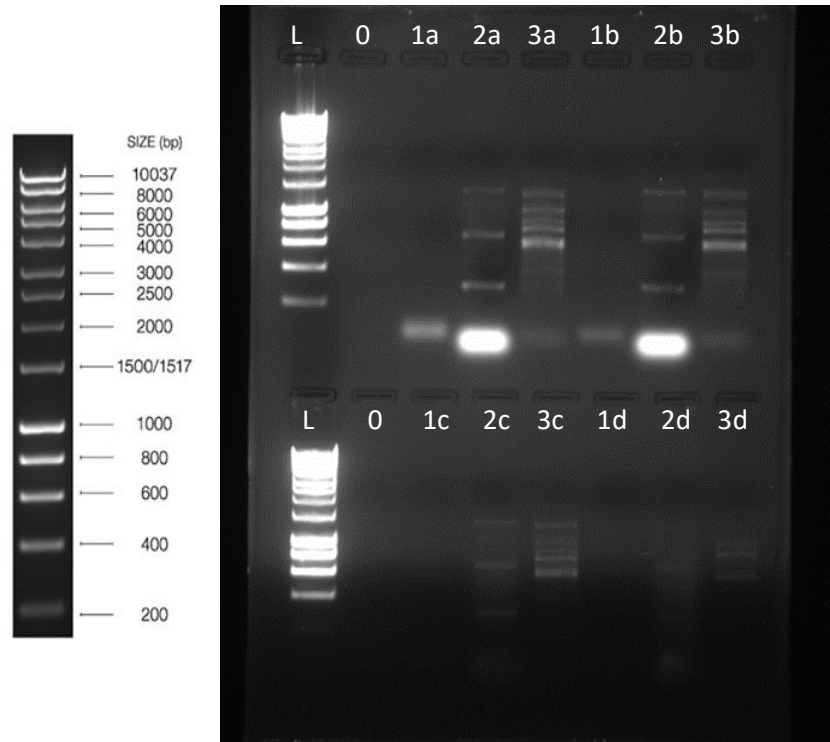


Figure 19. PCR resultant with gradient annealing temperatures and adjusted reaction mix. Letters indicate the temperature: a – 58°C, b – 59°C, c – 60.1°C and d – 62.5°C, number indicate the primer pair. 1 – COL1A2F1 and COL1A2R1, 2- COL1A2F2 and COL1A2R2, 3- COL1A2F3 and COL1A2R3. Negative control indicated as 0. Running conditions: 1.5% agarose, 99V, 35 minutes

temperature range for amplifying the target gene fragment of 58°C to 60°C. The resultant bands in Figure 19 sized over 3Kb are worth to be noticed which were the closest to the range of *COL1A2* genes discussed previously.

The annealing temperature of the products in Figures 19 were increased compared to the ones in applied in Figure 18, also the differences between these two attempts were their reaction mix with increased DNA polymerase and reduced volume of dNTP and DNA templates. The reaction mix recipe led to the result in Figure 18 was in each 25µL of reaction mix: 2.5µL of PCR buffer (10x), 1µL of dNTP, 0.2µL of *Taq* DNA polymerase, 0.5µL of DNA, 1µL of F primer, 1µL of R primer and 18.8µL of dH₂O. The one in

Figure 19 was in each of reaction mix: 2.5 μ L of PCR buffer (10x), 0.75 μ L of dNTP, 0.25 μ L of *Taq* DNA polymerase, 0.25 μ L of DNA and 1 μ L of each primer, topped up with distilled water to 25 μ L. The excessive banding determined the presence of non-specific bands which suggests that the PCR needed to be further optimisation. Due to time constraints further optimisation was not explored.

Chapter 4. Discussion

4.1 Collagen Extraction - Discussion

Collagen extraction methods with fish byproduct for research purpose are commonly conducted with acid extraction and pepsin enzyme extraction. In order to align the process to the industrial isinglass production, acid extraction was chosen in this project. Another factor influencing the decision to use acid extraction was the lack of previous studies on collagen extraction methods specifically from the swim bladder of *Arius maculatus*. Acid extraction was more suitable to start with for the exploratory nature of the protocol development for this specific species, as the acid extraction method is the most commonly employed technique in various tissue and species (Delgado et al., 2017; Li et al., 2013). The pepsin extraction methods had been popular and studied previously which benefits more in protein integrity (Skierka and Sadowska, 2007). The successful extraction of collagen using this method confirmed its feasibility for *Arius maculatus* swim bladders. Furthermore, the absence of enzymatic steps mirrors industrial isinglass production processes, making this approach more practical and cost-effective.

In this case, optimising the method was essential for the study of collagen from industrial pretreated fish maw from the *Arius Maculatus* or referred as catfish. In isinglass fining product from Murphy and Son's, there are two fish swim bladders from two different species of fish used as the raw material, considering the objective for the industry, *Arius Maculatus* was selected for study as this is one of the most commonly employed raw material in the industry. Optimising the extraction method for research purpose also enabled the characterisation such as the molecular size and

the patterns of the working collagen from catfish fish swim bladders which is absent in current available both research and industry database.

The choice of butanol is based on its effectiveness at removing fats from biological tissues. The rationale behind selecting a 10% concentration is to achieve an optimal balance between effective lipid removal and maintaining the structural integrity of the collagen. Enhanced lipid removal is essential not only for the purity of the collagen but also for its stability, as residual lipids can undergo oxidation, leading to degradation of the collagen matrix. Glacial acetic acid was prepared in two different dilutions: 0.5M and 0.1M. Acetic acid ensures minimal denaturation of the collagen structure due to its mild nature and higher yield compared to other acids (Skierka and Sadowska, 2007). The specific concentration of 0.5M was chosen based on numbers of studies on fish collagen proven on its optimised balance solubility and structural preservation, as higher concentrations may increase the risk of hydrolysis, leading to a decrease in molecular weight and potential loss of functional properties (Jafari et al., 2020).

The temperature of the extraction procedure needs to be at 4°C to prevent the collagen from denaturation and solidification to gelatine even the extraction not involving any temperature-sensitive enzyme. Secondly, the form of the raw samples impacts the extraction procedure by the dry and wet weight differences which should be an important consideration while adjusting the sample: acid ratio.

There was some of the precipitated sample was centrifuged at 4000x g for 110 minutes at 4°C with the Eppendorf™ Centrifuge 5810 R. The lower centrifuge speed was not the most ideal centrifuge condition; therefore, the centrifuge time was extended to remove as much as of the solution from the sample as possible.

This sponge-like texture of lyophilised collagen sample is because of the nature of collagen structural characteristics to form a unique scaffold which draws interests from the researchers doing biomedical studies in tissue engineering and healing adhesives.

The SDS-PAGE result provided fundamental information of the collagen from catfish. Collagen samples displayed some aggregation at the top of the gel, likely due to incomplete solubilisation or sample overloading. The gel composition may not have been optimal for separating high-molecular-weight proteins, suggesting the need for future optimisation. The result protein patterns match the result patterns of previous study on collagen

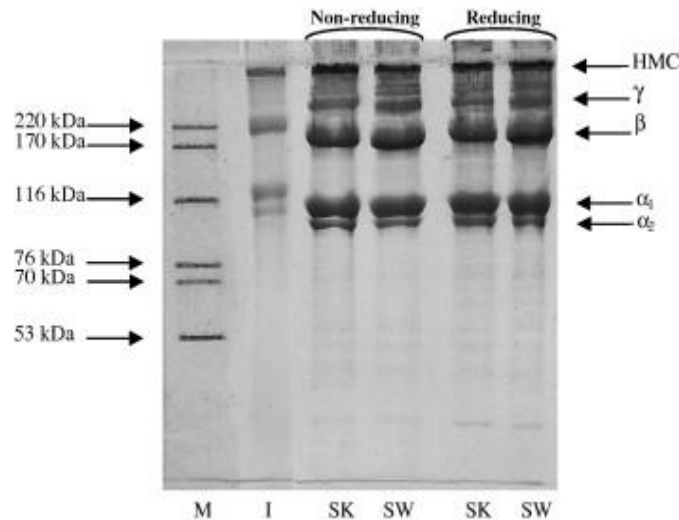


Figure 20. SDS-PAGE result from the protocol origin research article as reference to compare (Sinthusamran, Benjakul and Kishimura, 2013). Skin (SK) and swim bladder (SW) of seabass under non-reducing and reducing conditions. M- high molecular-weight protein markers, I - type I collagen from calf skin and HMC - high MW cross-linked components.

from fish byproduct from seabass (Figure 20). According to the studies and the composition observed from SDS-PAGE, the collagen from our sample shared the characteristics of type I collagen. The composition of the collagen molecule constituted by two α chains and the β -chain which had the molecular size of slightly over 115kDa and over 190kDa respectively.

The $\alpha 1$ and $\alpha 2$ chains was indicatable using the reference collagen SDS-PAGE result in Figure 20. Comparing the protein pattern under reducing conditions in Figure 13 to the non-reducing ones in Figure 11 and 12, there was no visible difference were determined under both conditions. This result suggested no presence of disulfide bonds as reducing agent contain dithiothreitol (DTT) which enable the disulfide bond reduction within the protein molecules (Lukesh et al., 2012).

For the positive control standard protein, the ideal collagen type I from calf fish skin was discontinued and was not able to purchase. The Fish Collagen Type I was purchased from Creative Enzymes® as substitute positive control as reference to the sample extracted. However, after a few of attempts with different concentration on the purchased standard collagen, there was no protein band at all with the product on SDS-PAGE. The exact molecular sizes need to be specified with adjustment on the SDS-PAGE gel with narrowed ranged matrix. As mentioned, techniques like western blotting can be employed in the identification and verification of collagen molecules in the future.

The fining test verified the laboratory-extracted collagen sample has the function as fining agent in beer as the commercial product. In future work, the clarity of the beer after adding the fining agent could be assessed alongside yeast cell count kits or a spectrophotometer to verify the results. In addition, due to the concentration of the collagen in commercial isinglass fining remains unspecified, an investigation on the collagen concentration can be carried out to provide a fairer comparison of the working efficiency in fining with the laboratory extracted collagen.

Further protein characterisation approaches such as amino acid mapping and protein sequences are encouraged to performed with both laboratory-extracted collagen from isinglass and purified commercial isinglass

products to provide more references for the recombinant isinglass or substitutes bacterial CLPs in the future.

4.2 DNA Extraction and Gene Isolation - Discussion

The choice of commercial DNA extraction kits was determined by their effectiveness in extracting DNA from a wide range of samples in laboratory settings or their proven success in previous studies focused on DNA extraction from fish swim bladders. All procedures were strictly followed as per the instructions provided with these kits. Both kits employed Proteinase K to degrade the protein of the sample tissue. In pursuit of optimising the extraction process, lysozyme was also experimented with, either as a replacement or as an additional enzyme in the extraction protocol.

Initial attempts with Qiagen DNeasy PowerSoil Kits and QIAamp DNA Mini kits encountered challenges in sample digestion and DNA degradation, as evidenced by gel electrophoresis results. There are several factors that were responsible for the failed DNA extraction using the commercial kits: 1. The floc form of swim bladders sample used in the extraction might retained polysaccharide residues, and since the sample was pretreated then dried, there might be some chemical residues remained which impact on the efficacy of kits extraction and the DNA purity. 2. Effective lysis is crucial for optimal DNA yield. Different samples have different cell structures, compositions, and properties, which can affect how well a particular enzyme or lysis method works, the effectiveness of the enzyme designed for a broad range of samples might vary when applied to unique sample types. In this case, the cell lysis incubation conditions including the enzyme with the kits might not have been ideal due to the nature of our sample. The size of the swim bladder in floc form might also not be ideal for kit extraction. 3. Kits are often designed for a range of sample types but may

not be optimised for specific tissues or organisms, affecting the efficiency of DNA extraction. The storage conditions and the age of the samples sometimes might lead to degradation or loss of DNA content during extraction using kits.

The challenge encountered in DNA extraction from catfish dry flocs highlight the limitations of both commercial kits in dealing with this particular form of samples. The difficulty and the inefficacy of enzymes in some conditions suggest that catfish swim bladder flocs present unique challenges that standard protocols may not address effectively. The observed DNA degradation points towards the need for optimised protocols that can protect DNA integrity during extraction.

The optimised manual extraction method found the necessity of salt precipitation for successful DNA extraction from flocs preserved in buffer. Sample DNA size was determined 10+Kb. The only genomic size information of *Arius Maculatus* was its mitochondrial DNA (mtDNA), which sized 16,710 bp in length (Yang et al., 2022), which can be valuable information taking as a consideration comparing and determining the DNA to the result in Figure 17.

The decision of switching to manual extraction methods, including phenol-chloroform extraction, was due by the poor performance of commercial kits. The manual method provided higher yield and better-quality DNA, as evidenced by the clear high-molecular-weight DNA bands observed on the gels after employed salt precipitation. The rationale for using lysozyme and salt precipitation was to effectively lyse effectively the dense swim bladder tissues and eliminated polysaccharides, which are common inhibitors in fish tissues (Kwak et al., 2003; Zhang et al., 2022). This optimised method approach contrasted with studies that utilised alternative enzymatic cocktails,

highlighting the species-specific challenges in DNA extraction (Lin et al., 2020).

The origin of the primers was well studied previously with known *COL1A2*-coding gene sequences utilised as relevant models in primers design to initiate *COL1A2* gene isolation from various vertebrates. Zebrafish collagen-coding gene has been popular in collagen studies due to its significant genetic similarities with other vertebrates (C. Gistelink et al., 2016). As zebrafish, the collagen type I from rainbow trout and chum salmon were featured in *COL1A2* gene research due to their well-studied molecular and structural characteristics (Berthelot et al., 2014; Matsui et al., 1991).

The *COL1A2* genes of the primers origins fishes have the sequence size from 4060bp (GenBank ID: AB075700) to 5182bp (GenBank ID: BC071278), and the *COL1A2* genes of grass carp in Yu et al., 2014 was 4899bp (GenBank ID: HM587241). These data can provide a rough range of 4000bp to 6000bp for target gene size, and as guides to locate the *COL1A2* gene within or around this range in the future.

Despite the PCR running conditions positively resulted in the original study, the initial attempts with the DNA sample in this case did not yield any desired results for the *COL1A2* genes. In response, several experimental adjustments were made in following attempts including varying the concentration of the DNA sample in the reaction mixture and employing a gradient PCR technique to identify the optimal annealing temperature. These adjustments were aimed at determining the optimal experimental procedures and enhancing the specificity and efficiency of the amplification process to isolate the desired collagen-coding genes from catfish swim bladders.

The issues with PCR amplification of collagen-coding genes underscore the need for precise optimisation of experimental conditions, including

annealing temperatures and master mix ratios, to achieve specific and clear results. The employment of gradient PCR provided the results of multiple non-specific bands under visualisations which could be the result of poor primer specificity or suboptimal reaction conditions. The primer set was selected for exploration purpose as the gap of collagen-coding gene research data on the swim bladders of *Arius Maculatus*.

The pair of primers diverted from zebrafish gave no result with PCRs under all conditions which means these primers might be not suitable for the *COL1A2* gene isolations with this species of fish under these experimented PCR conditions. The non-specific band resultants of other two pairs of primers indicated the needs of further optimisation of the PCR conditions, or the selection of alternative primers or primer cocktail might be necessary to achieve a more specific amplification and clearly identify the target gene fragment. Due to the time constraints of this project, the specific primers targeting this species collagen-coding gene was not able to explore and design, but it is achievable in future studies once more genetic information of DNA extract be further analysed.

Comparing these findings with existing literature could reveal gaps in knowledge about extracting DNA from similarly challenging samples and identifying specific genes. The novelty lies in its application to *Arius Maculatus*, a species underexplored at molecular level as its value as popular raw material for commercial isinglass production. Furthermore, this work optimised the essential foundational steps in future studies on this particular collagen from isinglass and the heterologous expression onwards pursuing the broader goal of sustainable fining agent production.

Chapter 5. Conclusion and Future Work

The optimised laboratory extraction methods revealed through acid solubilisation and the subsequent characterisation of collagen via SDS-PAGE affirm the structural integrity and functional viability of collagen from isinglass from the swim bladders of *Arius Maculatus*. Therefore, the collagen extracted using this method can be used in future and more detailed molecular characterisation to provide reference when finding alternatives for isinglass made from the swim bladder of both species of fish whether recombinant collagen or bacterial CLPs.

The optimised DNA extraction method from the floc form of swim bladder was successful. Despite the success of DNA extraction, PCR amplification presented notable challenges. While manual extraction methods improved DNA yield and quality for this specific tissue sample, non-specific amplification persisted, indicating a need for improved primer design and reaction optimisation which was left due to the time constraints. These limitations underscore the complexity of working with the underexplored molecular information on the swim bladders of the isinglass sourcing species like *Arius maculatus*.

Overall, this study provides foundational data and methodologies for future work on sustainable isinglass production. By addressing existing gaps in the literature, it provided an access for further exploration of recombinant collagen production.

5.1 Future Work

Further research is encouraged to refine these techniques and explore the full potential of recombinant and bacterial collagen-like proteins as viable substitutes for traditional isinglass, aiming for a balance between efficiency,

environmental sustainability, and consumer acceptability in the beverage industry.

The first step for the future work is suggested to be advance the protein analysis. Techniques such as Circular dichroism spectroscopy or known as CD, and Fourier-transform infrared spectroscopy (FTIR) can be employed to assess the secondary structure and stability of the extracted collagen from the swim bladders (Ramalingam et al., 2023). The advance protein analysis would be essential for building a future reference for both recombinant collagen and BCLP comparison and analysis.

To response to the challenges encountered during the PCR amplification, the specie-specific primers design using computational tool such as Primer3 and PCR optimisation are firmly encouraged in the future study on *Arius maculatus*. Due to the lack of genetic information of *Arius maculatus* that absent in past research, the genomic sequencing of the DNA extracted will be beneficial. Utilising available database like NCBI BLAST help to identify the potential collagen-coding sequence use related species of fishes' collagen-coding regions. In addition, finding an alternative high-fidelity polymerase with proofreading capabilities would potentially be able to reduce non-specific amplification (Karunanathie et al., 2022).

Once the collagen-coding gene be successfully isolated, the resultant can be sequenced and analysis to determine the protein coding region. To achieve heterologous expression, future studies should focus on codon optimisation for the collagen-coding genes and following with cloning and construction of plasmid vectors such as *pET* carrying the designated promotor. Heterologous expression can be performed with *E. coli* BL21(DE3) strain via chemical transformation (Green and Rogers, 2013) or via electroporation (Lessard, 2013) for small-scale expression trials to test the protein yielding and folding.

The long-term goal is to achieve heterologous expression of collagen from isinglass in microbial system. Once the heterologous expression succeeds, further advancement on the recombinant collagen including exploring scalable fermentation processes would lead to provide an allergen-free and environmentally sustainable alternatives to traditional isinglass.

Acknowledgement

This project was kindly fully funded by Murphy and Son Ltd. I would like to thank my supervisor Dr. Nagamani Bora for her support and invaluable supervisions during my MRes journey. My gratitude extends to Iain Kenny and Celina Dugulin from Murphy and Son's for their supports and industrial insights.

Reference List

- Ahmad, T., Ismail, A., Ahmad, S.A., Khalil, K.A., Kumar, Y., Adeyemi, K.D. and Sazili, A.Q. (2017). Recent advances on the role of process variables affecting gelatin yield and characteristics with special reference to enzymatic extraction: A review. *Food Hydrocolloids*, 63, pp.85–96. doi:<https://doi.org/10.1016/j.foodhyd.2016.08.007>.
- Alberghini, L., Truant, A., Santonicola, S., Colavita, G. and Giaccone, V. (2022). Microplastics in Fish and Fishery Products and Risks for Human Health: A Review. *International Journal of Environmental Research and Public Health*, 20(1), p.789. doi:<https://doi.org/10.3390/ijerph20010789>.
- Aly, N., Benoit, E., Chaubard, J.-L., Chintalapudi, K., Choung, S., de Leeuw, M., Diaz, M., Dueppen, D., Ferraro, B., Fischetti, V., Gassaway, E., Hansenne-Cervantes, I., Heeres, A., Karas, C., Khan, M., Kral, J.M., Lam, S., Lartey, R., Leonard, M. and Lue, S.W. (2022). Cosmetic Potential of a Recombinant 50 kDa Protein. *Cosmetics*, 9(1), p.8. doi:<https://doi.org/10.3390/cosmetics9010008>.
- Amirrah, I.N., Lokanathan, Y., Zulkiflee, I., Wee, M.F.M.R., Motta, A. and Fauzi, M.B. (2022). A Comprehensive Review on Collagen Type I Development of Biomaterials for Tissue Engineering: From Biosynthesis to Bioscaffold. *Biomedicines*, [online] 10(9), p.2307. doi:<https://doi.org/10.3390/biomedicines10092307>.
- An, B., Kaplan, D.L. and Brodsky, B. (2014). Engineered recombinant bacterial collagen as an alternative collagen-based biomaterial for tissue engineering. *Frontiers in Chemistry*, [online] 2. doi:<https://doi.org/10.3389/fchem.2014.00040>.
- Andersen, D.C. and Krummen, L. (2002). Recombinant protein expression for therapeutic applications. *Current Opinion in Biotechnology*, [online] 13(2), pp.117–123. doi:[https://doi.org/10.1016/S0958-1669\(02\)00300-2](https://doi.org/10.1016/S0958-1669(02)00300-2).
- Ar, G., Sm, B., Bm, V. and V, B. (2016). An overview of heterologous expression host systems for the production of recombinant proteins. *krishi.icar.gov.in*, [online] 4(7). Available at: <https://krishi.icar.gov.in/jspui/handle/123456789/9425>.
- Arakawa, T. and Timasheff, S.N. (1984). Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. *Biochemistry*, 23(25), pp.5912–5923. doi:<https://doi.org/10.1021/bi00320a004>.
- Avila Rodríguez, M.I., Rodríguez Barroso, L.G. and Sánchez, M.L. (2017). Collagen: A review on its sources and potential cosmetic applications. *Journal of Cosmetic Dermatology*, [online] 17(1), pp.20–26. doi:<https://doi.org/10.1111/jocd.12450>.
- Bamforth, C.W. (2018). Beer Haze. *Journal of the American Society of Brewing Chemists*, [online] 57(3). doi:<https://doi.org/10.1094/ASBCJ-57-0081>.

- Barnes, L.M., Bentley, C.M. and Dickson, A.J. (2003). Stability of protein production from recombinant mammalian cells. *Biotechnology and Bioengineering*, 81(6), pp.631–639. doi:<https://doi.org/10.1002/bit.10517>.
- Bella, J. and Hulmes, D.J.S. (2017). Fibrillar Collagens. *Subcellular Biochemistry*, 82, pp.457–490. doi:https://doi.org/10.1007/978-3-319-49674-0_14.
- Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.-M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H. and Boussaha, M. (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nature Communications*, [online] 5. doi:<https://doi.org/10.1038/ncomms4657>.
- Bhadra, B., Sakpal, A., Patil, S., Patil, S., Date, A., Prasad, V. and Dasgupta, S. (2021). A Guide to Collagen Sources, Applications and Current Advancements. *Systematic Bioscience and Engineering*, pp.67–87. doi:<https://doi.org/10.37256/sbe.1220211043>.
- Bourdon, B., Frédéric Cassé, Gruchy, N., Cambier, P., Leclercq, S., Oddoux, S., Noël, A., Lafont, J., Romain Contentin and Philippe Galéra (2021). Marine Collagen Hydrolysates Promote Collagen Synthesis, Viability and Proliferation While Downregulating the Synthesis of Pro-Catabolic Markers in Human Articular Chondrocytes. *International Journal of Molecular Sciences*, [online] 22(7), pp.3693–3693. doi:<https://doi.org/10.3390/ijms22073693>.
- Brodsky, B. and Ramshaw, J.A.M. (2017). Bioengineered Collagens. *Subcellular Biochemistry*, pp.601–629. doi:https://doi.org/10.1007/978-3-319-49674-0_18.
- Buckley, M.R., Evans, E.B., Matuszewski, P.E., Chen, Y.-L., Satchel, L.N., Elliott, D.M., Soslowsky, L.J. and Dodge, G.R. (2013). Distributions of types I, II and III collagen by region in the human supraspinatus tendon. *Connective Tissue Research*, 54(6), pp.374–379. doi:<https://doi.org/10.3109/03008207.2013.847096>.
- Buechter, D.D., Paoletta, D.N., Leslie, B.S., Brown, M.S., Mehos, K.A. and Gruskin, E.A. (2003). Co-translational Incorporation of Trans-4-Hydroxyproline into Recombinant Proteins in Bacteria. *Journal of Biological Chemistry*, 278(1), pp.645–650. doi:<https://doi.org/10.1074/jbc.m209364200>.
- Bulleid, N.J., John, D.C.A. and Kadler, K.E. (2000). Recombinant expression systems for the production of collagen. *Biochemical Society Transactions*, 28(4), pp.350–353. doi:<https://doi.org/10.1042/bst0280350>.
- C. Gistelink, Gioia, R., Gagliardi, A., Tonelli, F., Marchese, L., Bianchi, L., Landi, C., Bini, L., A. Huysseune, Witten, P.E., Staes, A., K. Gevaert, N. De Rocker, Menten, B., F. Malfait, S. Leikin, Carra, S., Ruggero Tenni, Rossi, A. and A. De Paepe (2016). Zebrafish Collagen Type I: Molecular and Biochemical Characterization of the

Major Structural Protein in Bone and Skin. *Scientific Reports*, [online] 6(1). doi:<https://doi.org/10.1038/srep21540>.

Cimini, A. and Moresi, M. (2017). Towards a Kieselguhr- and PVPP-Free Clarification and Stabilization Process of Rough Beer at Room-Temperature Conditions. *Journal of Food Science*, 83(1), pp.129–137. doi:<https://doi.org/10.1111/1750-3841.13989>.

Conway, J. (2023). *Beer consumption in the UK 2008-2019* | Statista. [online] Statista. Available at: <https://www.statista.com/statistics/446838/volume-beer-consumption-united-kingdom-uk/>.

Cosme, F., Ricardo-da-Silva, J.M. and Laureano, O. (2009). Effect of Various Proteins on Different Molecular Weight Proanthocyanidin Fractions of Red Wine during Wine Fining. *American Journal of Enology and Viticulture*, [online] 60(1), pp.74–81. doi:<https://doi.org/10.5344/ajev.2009.60.1.74>.

De Luca, C., Mikhal'chik, E.V., Suprun, M.V., Papacharalambous, M., Truhanov, A.I. and Korkina, L.G. (2016). Skin Antiageing and Systemic Redox Effects of Supplementation with Marine Collagen Peptides and Plant-Derived Antioxidants: A Single-Blind Case-Control Clinical Study. *Oxidative Medicine and Cellular Longevity*, [online] 2016. doi:<https://doi.org/10.1155/2016/4389410>.

Delgado, L.M., Shologu, N., Fuller, K. and Zeugolis, D.I. (2017). Acetic acid and pepsin result in high yield, high purity and low macrophage response collagen for biomedical applications. *Biomedical Materials*, 12(6), p.065009. doi:<https://doi.org/10.1088/1748-605x/aa838d>.

Deyl, Z., Mikšíkl. and Eckhardt, A. (2003). Preparative procedures and purity assessment of collagen proteins. *Journal of Chromatography B*, 790(1-2), pp.245–275. doi:[https://doi.org/10.1016/s1570-0232\(03\)00158-2](https://doi.org/10.1016/s1570-0232(03)00158-2).

Dong, Y. and Dai, Z. (2022). Physicochemical, Structural and Antioxidant Properties of Collagens from the Swim Bladder of Four Fish Species. *Marine Drugs*, 20(9), pp.550–550. doi:<https://doi.org/10.3390/md20090550>.

Exposito, J.-Y., Valcourt, U., Cluzel, C. and Lethias, C. (2010). The Fibrillar Collagen Family. *International Journal of Molecular Sciences*, 11(2), pp.407–426. doi:<https://doi.org/10.3390/ijms11020407>.

Felician, F.F., Xia, C., Qi, W. and Xu, H. (2018). Collagen from Marine Biological Sources and Medical Applications. *Chemistry & Biodiversity*, 15(5), p.e1700557. doi:<https://doi.org/10.1002/cbdv.201700557>.

Fu, C., Shi, S., Wei, N., Fan, Y., Gu, H., Liu, P. and Xiao, J. (2023). Biocompatible Triple-Helical Recombinant Collagen Dressings for Accelerated Wound Healing in Microneedle-Injured and Photodamaged Skin. *Cosmetics*, [online] 10(1), p.31. doi:<https://doi.org/10.3390/cosmetics10010031>.

Gadon, A.G. (2020). *A novel beer fining agent extracted from hops*. [online] eprints.nottingham.ac.uk. Available at: <https://eprints.nottingham.ac.uk/61144/>.

Gomes, A.R., Byregowda, S.M., Veeregowda, B.M. and Balamurugan, V. (2016). An Overview of Heterologous Expression Host Systems for the Production of Recombinant Proteins. *Advances in Animal and Veterinary Sciences*, 4(7), pp.346–356. doi:<https://doi.org/10.14737/journal.aavs/2016/4.7.346.356>.

Green, R. and Rogers, E.J. (2013). Transformation of chemically competent E. coli. *Methods in Enzymology*, [online] 529, pp.329–336. doi:<https://doi.org/10.1016/B978-0-12-418687-3.00028-8>.

Gupta, S.K. and Shukla, P. (2015). Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications. *Critical Reviews in Biotechnology*, 36(6), pp.1089–1098. doi:<https://doi.org/10.3109/07388551.2015.1084264>.

He, X., Xie, L., Zhang, X., Lin, F., Wen, X. and Teng, B. (2021). The Structural Characteristics of Collagen in Swim Bladders with 25-Year Sequence Aging: The Impact of Age. *Applied Sciences*, 11(10), p.4578. doi:<https://doi.org/10.3390/app11104578>.

Hickman, D., Sims, T.J., Miles, C.A., Bailey, A.J., de Mari, M. and Koopmans, M. (2000). Isinglass/collagen: denaturation and functionality. *Journal of Biotechnology*, [online] 79(3), pp.245–257. doi:[https://doi.org/10.1016/S0168-1656\(00\)00241-8](https://doi.org/10.1016/S0168-1656(00)00241-8).

И.Ф. Горлов, Titov, E., Семенов, Г.Б., Slozhenkina, M.I., Aleksandr Yurievich Sokolov, Ruslan Omarov, Aleksandr Ivanovich Goncharov, Elena Yurievna Zlobina, Литвинова, Е.В. and Karpenko, E.V. (2018). Collagen from porcine skin: a method of extraction and structural properties. *International Journal of Food Properties*, 21(1), pp.1031–1042. doi:<https://doi.org/10.1080/10942912.2018.1466324>.

Isaac, A. and Lawson, A. (2023). Revealed: Sellafield nuclear site has leak that could pose risk to public. *The Guardian*. [online] 5 Dec. Available at: <https://www.theguardian.com/business/2023/dec/05/sellafield-nuclear-site-leak-could-pose-risk-to-public> [Accessed 11 Dec. 2023].

Isinglass as a Substitute for Human Blood Plasma. (1944). *Nature*, 153(3878), pp.247–248. doi:<https://doi.org/10.1038/153247d0>.

Jafari, H., Lista, A., Siekapen, M.M., Ghaffari-Bohlouli, P., Nie, L., Alimoradi, H. and Shavandi, A. (2020). Fish Collagen: Extraction, Characterization, and Applications for Biomaterials Engineering. *Polymers*, 12(10), p.2230. doi:<https://doi.org/10.3390/polym12102230>.

Jarita Duasa, Afifah Muhamad Husin, Asmy, M. and Maya Puspa Rahman (2021). An alternative source of collagen for Muslim consumers: *halal* and environmental

concerns. *Journal of Islamic Marketing*, 13(11), pp.2232–2253. doi:<https://doi.org/10.1108/jima-09-2020-0268>.

Jeevithan, E., Bao, B., Bu, Y., Zhou, Y., Zhao, Q. and Wu, W. (2014). Type II Collagen and Gelatin from Silvertip Shark (*Carcharhinus albimarginatus*) Cartilage: Isolation, Purification, Physicochemical and Antioxidant Properties. *Marine Drugs*, [online] 12(7), pp.3852–3873. doi:<https://doi.org/10.3390/md12073852>.

Jeżowski, P. and Kowalczewski, P.Ł. (2023). Isinglass as an Alternative Biopolymer Membrane for Green Electrochemical Devices: Initial Studies of Application in Electric Double-Layer Capacitors and Future Perspectives. *Polymers*, [online] 15(17), p.3557. doi:<https://doi.org/10.3390/polym15173557>.

Kananavičiūtė, R., Kvederavičiūtė, K., Dabkevičienė, D., Mackevičius, G. and Kuisienė, N. (2020). Collagen-like sequences encoded by extremophilic and extremotolerant bacteria. *Genomics*, 112(3), pp.2271–2281. doi:<https://doi.org/10.1016/j.ygeno.2019.12.023>.

Karunanathie, H., Kee, P.S., Ng, S.F., Kennedy, M.A. and Chua, E.W. (2022). PCR enhancers: Types, mechanisms, and applications in long-range PCR. *Biochimie*, [online] 197, pp.130–143. doi:<https://doi.org/10.1016/j.biochi.2022.02.009>.

Kaur, J., Kumar, A. and Kaur, J. (2018). Strategies for optimization of heterologous protein expression in *E. coli* : Roadblocks and reinforcements. *International Journal of Biological Macromolecules*, [online] 106, pp.803–822. doi:<https://doi.org/10.1016/j.ijbiomac.2017.08.080>.

Khan, S., Ullah, M.W., Siddique, R., Nabi, G., Manan, S., Yousaf, M. and Hou, H. (2016). Role of Recombinant DNA Technology to Improve Life. *International Journal of Genomics*, [online] 2016(2405954), pp.1–14. doi:<https://doi.org/10.1155/2016/2405954>.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. (2010). Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*). *LWT - Food Science and Technology*, 43(5), pp.792–800. doi:<https://doi.org/10.1016/j.lwt.2010.01.006>.

Kwak, J. -K, Park, S.W., Koo, J. -G, Cho, M. -G, Buchholz, R. and Goetz, P. (2003). Enhancement of the Non-Specific Defence Activities in Carp (*Cyprinus carpio*) and Flounder (*Paralichthys olivaceus*) by Oral Administration of Schizophyllan. *Acta Biotechnologica*, 23(4), pp.359–371. doi:<https://doi.org/10.1002/abio.200390046>.

LAEMMLI, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227(5259), pp.680–685. doi:<https://doi.org/10.1038/227680a0>.

- Leach, A.A. (1967). COLLAGEN CHEMISTRY IN RELATION TO ISINGLASS AND ISINGLASS FININGS-A REVIEW. *Journal of the Institute of Brewing*, 73(1), pp.8–16. doi:<https://doi.org/10.1002/j.2050-0416.1967.tb03011.x>.
- Lee, H., Yang, G.H., Kim, M., Lee, J., Huh, J. and Kim, G. (2018). Fabrication of micro/nanoporous collagen/dECM/silk-fibroin biocomposite scaffolds using a low temperature 3D printing process for bone tissue regeneration. *Materials Science and Engineering: C*, 84, pp.140–147. doi:<https://doi.org/10.1016/j.msec.2017.11.013>.
- Leiper, K.A., Duszanskyj, R. and Stewart, G.G. (2002). Premixing of Isinglass and Silica Gel to Obtain Improved Beer Stability. *Journal of the Institute of Brewing*, 108(1), pp.28–31. doi:<https://doi.org/10.1002/j.2050-0416.2002.tb00117.x>.
- Lessard, J.C. (2013). Transformation of E. coli Via Electroporation. *Methods in Enzymology*, pp.321–327. doi:<https://doi.org/10.1016/b978-0-12-418687-3.00027-6>.
- Li, Z.-R., Wang, B., Chi, C., Zhang, Q.-H., Gong, Y., Tang, J.-J., Luo, H. and Ding, G. (2013). Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorus niphonius*). *Food Hydrocolloids*, 31(1), pp.103–113. doi:<https://doi.org/10.1016/j.foodhyd.2012.10.001>.
- Lim, Y.-S., Ok, Y.-J., Hwang, S.-Y., Kwak, J.-Y. and Yoon, S. (2019). Marine Collagen as A Promising Biomaterial for Biomedical Applications. *Marine Drugs*, 17(8), p.467. doi:<https://doi.org/10.3390/md17080467>.
- Liu, D., Nikoo, M., Boran, G., Zhou, P. and Regenstein, J.M. (2015a). Collagen and gelatin. *Annual review of food science and technology*, [online] 6, pp.527–57. doi:<https://doi.org/10.1146/annurev-food-031414-111800>.
- Liu, D., Wei, G., Li, T., Hu, J., Lu, N., Regenstein, J.M. and Zhou, P. (2015b). Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin. *Food Chemistry*, 172, pp.836–843. doi:<https://doi.org/10.1016/j.foodchem.2014.09.147>.
- Lukesh, J.C., Palte, M.J. and Raines, R.T. (2012). A Potent, Versatile Disulfide-Reducing Agent from Aspartic Acid. *Journal of the American Chemical Society*, 134(9), pp.4057–4059. doi:<https://doi.org/10.1021/ja211931f>.
- Mahin Pouresmaeil and Shahnam Azizi-Dargahlou (2023). Factors involved in heterologous expression of proteins in E. coli host. *Arch Microbiol*, 205(5). doi:<https://doi.org/10.1007/s00203-023-03541-9>.
- Marchal, R. and Waters, E.J. (2010). New directions in stabilization, clarification and fining of white wines. *Managing Wine Quality*, pp.188–225. doi:<https://doi.org/10.1533/9781845699987.1.188>.

- Marques, C.F., Diogo, G.S., Pina, S., Oliveira, J.M., Silva, T.H. and Reis, R.L. (2019). Collagen-based bioinks for hard tissue engineering applications: a comprehensive review. *Journal of Materials Science: Materials in Medicine*, 30(3). doi:<https://doi.org/10.1007/s10856-019-6234-x>.
- Marques, L. (2020). Collapse of Biodiversity in the Aquatic Environment. *Springer eBooks*, pp.275–301. doi:https://doi.org/10.1007/978-3-030-47527-7_11.
- Mastanjević, K., Krstanović, V., Lukinac, J., Jukić, M., Vulin, Z. and Mastanjević, K. (2018). Beer–The Importance of Colloidal Stability (Non-Biological Haze). *Fermentation*, 4(4), p.91. doi:<https://doi.org/10.3390/fermentation4040091>.
- Matsui, R., Ishida, M. and Kimura, S. (1991). Characterization of an $\alpha 3$ chain from the skin type I collagen of chum salmon (*Oncorhynchus keta*). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 99(1), pp.171–174. doi:[https://doi.org/10.1016/0305-0491\(91\)90025-9](https://doi.org/10.1016/0305-0491(91)90025-9).
- Murphy and Son. (n.d.). *Function of Isinglass Finings*. [online] Available at: <https://www.murphyandson.co.uk/resources/technical-articles/function-of-isinglass-finings/> [Accessed 5 Jan. 2024].
- Naomi, R., Ridzuan, P.M. and Bahari, H. (2021). Current Insights into Collagen Type I. *Polymers*, [online] 13(16). doi:<https://doi.org/10.3390/polym13162642>.
- National Center for Biotechnology Information (2017). *Polymerase Chain Reaction (PCR)*. [online] Nih.gov. Available at: <https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/>.
- Nogrady, B. (2023). Is Fukushima wastewater release safe? What the science says. *Nature*, [online] 618(7967), pp.894–895. doi:<https://doi.org/10.1038/d41586-023-02057-y>.
- Nugawela, N.P.P.S., Mahaliyana, A.S., Abhiram, G. and Abeygunawardena, A.P. (2023). A meta-analytic review of microplastic pollution in the Indian Ocean: Ecological health and seafood safety risk implications. *Marine Pollution Bulletin*, [online] 193, p.115213. doi:<https://doi.org/10.1016/j.marpolbul.2023.115213>.
- Olsen, D. (2003). Recombinant collagen and gelatin for drug delivery. *Advanced Drug Delivery Reviews*, 55(12), pp.1547–1567. doi:<https://doi.org/10.1016/j.addr.2003.08.008>.
- Ongley, S.E., Bian, X., Neilan, B.A. and Müller, R. (2013). Recent advances in the heterologous expression of microbial natural product biosynthetic pathways. *Natural Product Reports*, 30(8), p.1121. doi:<https://doi.org/10.1039/c3np70034h>.
- Pacak, C.A., MacKay, A.A. and Cowan, D.B. (2014). An Improved Method for the Preparation of Type I Collagen From Skin. *Journal of Visualized Experiments*, (83). doi:<https://doi.org/10.3791/51011>.

Pacak, C.A., Powers, J.M. and Cowan, D.B. (2011). Ultrarapid purification of collagen type I for tissue engineering applications. *Tissue Engineering. Part C, Methods*, [online] 17(9), pp.879–885. doi:<https://doi.org/10.1089/ten.TEC.2010.0720>.

Parry, D.A.D. and Squire, J.M. (2017). *Fibrous Proteins: Structures and Mechanisms*. Cham Springer International Publishing Springer, pp.601–629.

Paterson, G.K., Nieminen, L., Johanna M.C. Jefferies and Mitchell, T.J. (2008). PclA, a pneumococcal collagen-like protein with selected strain distribution, contributes to adherence and invasion of host cells. *FEMS Microbiology Letters*, 285(2), pp.170–176. doi:<https://doi.org/10.1111/j.1574-6968.2008.01217.x>.

Peebo, K. and Neubauer, P. (2018). Application of Continuous Culture Methods to Recombinant Protein Production in Microorganisms. *Microorganisms*, 6(3), p.56. doi:<https://doi.org/10.3390/microorganisms6030056>.

Peng, Y.Y., Howell, L., Stoichevska, V., Werkmeister, J.A., Dumsday, G.J. and Ramshaw, J.A.M. (2012). Towards scalable production of a collagen-like protein from *Streptococcus pyogenes* for biomedical applications. *Microbial Cell Factories*, 11(1). doi:<https://doi.org/10.1186/1475-2859-11-146>.

Peng, Y.Y., Stoichevska, V., Madsen, S., Howell, L., Dumsday, G.J., Werkmeister, J.A. and Ramshaw, J.A.M. (2014). A simple cost-effective methodology for large-scale purification of recombinant non-animal collagens. *Applied Microbiology and Biotechnology*, 98(4), pp.1807–1815. doi:<https://doi.org/10.1007/s00253-013-5475-8>.

Peng, Y.Y., Stoichevska, V., Vashi, A., Howell, L., Fehr, F., Dumsday, G.J., Werkmeister, J.A. and Alan, J. (2015). Non–animal collagens as new options for cosmetic formulation. *International Journal of Cosmetic Science*, 37(6), pp.636–641. doi:<https://doi.org/10.1111/ics.12243>.

Petukhova, T. (1998). *Potential Applications of Isinglass Adhesive for Paper Conservation*. [online] cool.culturalheritage.org. Available at: <https://cool.culturalheritage.org/coolaic/sg/bpg/annual/v08/bp08-06.html>.

Picker, J., Lan, Z., Arora, S., Green, M., Hahn, M.S., Cosgriff-Hernández, E. and Magnus Höök (2022). Prokaryotic Collagen-Like Proteins as Novel Biomaterials. *Frontiers in Bioengineering and Biotechnology*, 10. doi:<https://doi.org/10.3389/fbioe.2022.840939>.

Pinkas, D.M., Ding, S., Raines, R.T. and Barron, A.E. (2011). Tunable, Post-translational Hydroxylation of Collagen Domains in *Escherichia coli*. *ACS Chemical Biology*, 6(4), pp.320–324. doi:<https://doi.org/10.1021/cb100298r>.

Promega (2019). *DNA Purification | DNA Extraction Methods | Promega*. [online] Promega.co.uk. Available at:

<https://www.promega.co.uk/resources/guides/nucleic-acid-analysis/dna-purification/>.

Qi Jue Chen, Pei, Y., Tang, K. and Madalina Georgiana Albu-Kaya (2023). Structure, extraction, processing, and applications of collagen as an ideal component for biomaterials - a review. *Collagen and Leather*, 5(1). doi:<https://doi.org/10.1186/s42825-023-00127-5>.

Qi, Y., Zhou, D., Kessler, J.L., Qiu, R., Yu, S.M., Li, G., Qin, Z. and Li, Y. (2022). Terminal repeats impact collagen triple-helix stability through hydrogen bonding. *Chemical Science*, [online] 13(42), pp.12567–12576. doi:<https://doi.org/10.1039/D2SC03666E>.

Rai, M. and Padh, H. (2001). Expression systems for production of heterologous proteins. *Current Science*, [online] 80(9), pp.1121–1128. Available at: <https://www.jstor.org/stable/24105768?seq=1>.

Ramalingam, M., Balasubramani Govindasamy, Sumit, R., Suresh Ayothi and Meena Boominathan (2023). Purification and characterization of metallothionein protein in marine catfish, *Arius arius*, on exposure to cadmium. *Biomass Conversion and Biorefinery*, 14(17), pp.21095–21105. doi:<https://doi.org/10.1007/s13399-023-04483-4>.

Ramshaw, J.A., Werkmeister, J.A. and Dumsday, G.J. (2014). Bioengineered collagens. *Bioengineered*, 5(4), pp.227–233. doi:<https://doi.org/10.4161/bioe.28791>.

Rasmussen, M., Jacobsson, M. and Björck, L. (2003). Genome-based Identification and Analysis of Collagen-related Structural Motifs in Bacterial and Viral Proteins. *Journal of Biological Chemistry*, 278(34), pp.32313–32316. doi:<https://doi.org/10.1074/jbc.m304709200>.

Rong, H., Lin, F., Ning, L., Wu, K., Chen, B., Zheng, J., Samwel Mchele Limbu and Wen, X. (2022). Cloning, tissue distribution and mRNA expression of type I collagen alpha 1 gene from Chu's croaker (*Nibea coibor*). *Gene*, 824, pp.146441–146441. doi:<https://doi.org/10.1016/j.gene.2022.146441>.

Rosano, G.L. and Ceccarelli, E.A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*, [online] 5(172). doi:<https://doi.org/10.3389/fmicb.2014.00172>.

Ryder, D.S. (2017). Processing Aids in Brewing*. *CRC Press eBooks*, pp.287–328. doi:<https://doi.org/10.1201/9781351228336-10>.

Sadovy de Mitcheson, Y., To, A.W., Wong, N.W., Kwan, H.Y. and Bud, W.S. (2019). Emerging from the murk: threats, challenges and opportunities for the global swim bladder trade. *Reviews in Fish Biology and Fisheries*, 29(4), pp.809–835. doi:<https://doi.org/10.1007/s11160-019-09585-9>.

- Saito, M., Takenouchi, Y., Kunisaki, N. and Kimura, S. (2001). Complete primary structure of rainbow trout type I collagen consisting of $\alpha 1(I)\alpha 2(I)\alpha 3(I)$ heterotrimers. *European Journal of Biochemistry*, 268(10), pp.2817–2827. doi:<https://doi.org/10.1046/j.1432-1327.2001.02160.x>.
- Santos, M.H., Silva, R.M., Dumont, V.C., Neves, J.S., Mansur, H.S. and Heneine, L.G.D. (2013). Extraction and characterization of highly purified collagen from bovine pericardium for potential bioengineering applications. *Materials Science and Engineering: C*, [online] 33(2), pp.790–800. doi:<https://doi.org/10.1016/j.msec.2012.11.003>.
- Sarkar, Md.S.I., Hasan, M.M., Hossain, Md.S., Khan, M., Islam, A.A., Paul, S.K., Rasul, Md.G. and Kamal, Md. (2023a). Exploring fish in a new way: A review on non-food industrial applications of fish. *Heliyon*, [online] 9(12), p.e22673. doi:<https://doi.org/10.1016/j.heliyon.2023.e22673>.
- Sarkar, Md.S.I., Hasan, M.M., Hossain, Md.S., Khan, M., Islam, A.A., Paul, S.K., Rasul, Md.G. and Kamal, Md. (2023b). Exploring fish in a new way: A review on non-food industrial applications of fish. *Heliyon*, [online] 9(12), p.e22673. doi:<https://doi.org/10.1016/j.heliyon.2023.e22673>.
- Schmidt, M., Dornelles, Mello, R., Mazutti, M. and Demiate, I. (2016). Collagen extraction process. *International Food Research Journal*, 23(3), pp.913–922.
- Senadheera, T.R.L., Dave, D. and Shahidi, F. (2020). Sea Cucumber Derived Type I Collagen: A Comprehensive Review. *Marine Drugs*, 18(9), p.471. doi:<https://doi.org/10.3390/md18090471>.
- Shenoy, M., Abdul, N.S., Qamar, Z., Bahri, B.M.A., Al Ghalayini, K.Z.K. and Kakti, A. (2022). Collagen Structure, Synthesis, and Its Applications: A Systematic Review. *Cureus*. doi:<https://doi.org/10.7759/cureus.24856>.
- Shoulders, M.D. and Raines, R.T. (2009). Collagen Structure and Stability. *Annual Review of Biochemistry*, [online] 78(1), pp.929–958. doi:<https://doi.org/10.1146/annurev.biochem.77.032207.120833>.
- Sibilla, S., Godfrey, M., Brewer, S., Budh-Raja, A. and Genovese, L. (2015). An Overview of the Beneficial Effects of Hydrolysed Collagen as a Nutraceutical on Skin Properties: Scientific Background and Clinical Studies. *The Open Nutraceuticals Journal*, [online] 8(1). Available at: <https://benthamopen.com/ABSTRACT/TONUTRAJ-8-29>.
- Sinthusamran, S., Benjakul, S. and Kishimura, H. (2013). Comparative study on molecular characteristics of acid soluble collagens from skin and swim bladder of seabass (*Lates calcarifer*). *Food Chemistry*, [online] 138(4), pp.2435–2441. doi:<https://doi.org/10.1016/j.foodchem.2012.11.136>.
- Skierka, E. and Sadowska, M. (2007). The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*). *Food*

Chemistry, [online] 105(3), pp.1302–1306.
doi:<https://doi.org/10.1016/j.foodchem.2007.04.030>.

Smith, B.J. (1984). SDS Polyacrylamide Gel Electrophoresis of Proteins. *Humana Press*, [online] 1, pp.41–56. doi:<https://doi.org/10.1385/0-89603-062-8:41>.

Smith, M. (2024). *Polymerase Chain Reaction (PCR)*. [online] Genome.gov. Available at: <https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction>.

Smith, M., Love, D.C., Rochman, C.M. and Neff, R.A. (2018). Microplastics in Seafood and the Implications for Human Health. *Current Environmental Health Reports*, [online] 5(3), pp.375–386. doi:<https://doi.org/10.1007/s40572-018-0206-z>.

Soheila Naderi Gharahgheshlagh, Latifi, N., Ghadimi, T., Siamak Farokh Forghani, Irilouzadian, R., Amini, N., Ghazaleh Larijani, Hatami, S., Negar Taghavian, Sina Bayat Shahbazi and Latifi, F. (2023). Biochemical and Biological Characterization of Type-I Collagen from *Scomberomorus commerson* Skin as a Biomaterial for Medical Applications. *International Journal of Peptide Research and Therapeutics*, 29(4). doi:<https://doi.org/10.1007/s10989-023-10534-1>.

Sonal Gahlawat, Nanda, V. and Shreiber, D.I. (2023). Purification of recombinant bacterial collagens containing structural perturbations. *PLOS ONE*, 18(5), pp.e0285864–e0285864. doi:<https://doi.org/10.1371/journal.pone.0285864>.

Sunday, A. (2018). Effect of clarifying agents(Gelatin and Kaolin) on fruit wine productio. [online] 6(4). Available at: https://www.researchgate.net/publication/323607107_Effect_of_clarifying_agentsGelatin_and_Kaolin_on_fruit_wine_production.

Sylvestre, P., Couture-Tosi, E. and Mock, M. (2002). A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Molecular Microbiology*, 45(1), pp.169–178. doi:<https://doi.org/10.1046/j.1365-2958.2000.03000.x>.

Tarnutzer, K., Siva Sankar, D., Dengjel, J. and Ewald, C.Y. (2023). Collagen constitutes about 12% in females and 17% in males of the total protein in mice. *Scientific Reports*, [online] 13(1), p.4490. doi:<https://doi.org/10.1038/s41598-023-31566-z>.

Todd, S.J., Moir, A.J.G., Johnson, M.J. and Moir, A. (2003). Genes of *Bacillus cereus* and *Bacillus anthracis* Encoding Proteins of the Exosporium. *Journal of Bacteriology*, 185(11), pp.3373–3378. doi:<https://doi.org/10.1128/jb.185.11.3373-3378.2003>.

USD Analytics (2024). *Isinglass Market*. [online] Usdanalytics.com. Available at: <https://www.usdanalytics.com/industry-reports/isinglass-market>.

- Van Der Rest, M. and Garrone, R. (1991). Collagen family of proteins. *The FASEB Journal*, 5(13), pp.2814–2823. doi:<https://doi.org/10.1096/fasebj.5.13.1916105>.
- Vassilopoulou, E., Karathanos, A., Siragakis, G., Giavi, S., Sinaniotis, A., Douladiris, N., Fernandez-Rivas, M., Clausen, M. and Papadopoulos, N.G. (2011). Risk of allergic reactions to wine, in milk, egg and fish-allergic patients. *Clinical and Translational Allergy*, 1(1), p.10. doi:<https://doi.org/10.1186/2045-7022-1-10>.
- Vieira Gomes, A., Souza Carmo, T., Silva Carvalho, L., Mendonça Bahia, F. and Parachin, N. (2018). Comparison of Yeasts as Hosts for Recombinant Protein Production. *Microorganisms*, 6(2), p.38. doi:<https://doi.org/10.3390/microorganisms6020038>.
- Walker, S.L., Camarena, M.C.D. and Freeman, G. (2007). Alternatives to Isinglass for Beer Clarification. *Journal of the Institute of Brewing*, [online] 113(4), pp.347–354. doi:<https://doi.org/10.1002/j.2050-0416.2007.tb00761.x>.
- Wang, H. (2021). A Review of the Effects of Collagen Treatment in Clinical Studies. *Polymers*, [online] 13(22), p.3868. doi:<https://doi.org/10.3390/polym13223868>.
- Wang, T., Lew, J., Premkumar, J., Poh, C.L. and Win Naing, M. (2017). Production of recombinant collagen: state of the art and challenges. *Engineering Biology*, 1(1), pp.18–23. doi:<https://doi.org/10.1049/enb.2017.0003>.
- Wang, Y., Wang, Z. and Dong, Y. (2023). Collagen-Based Biomaterials for Tissue Engineering. *ACS Biomaterials Science & Engineering*. doi:<https://doi.org/10.1021/acsbiomaterials.2c00730>.
- Ward, I. (n.d.). *WORT & BEER CLARIFICATION MANUAL*.
- Wetterstrand, K. (2022). *Recombinant DNA technology*. [online] National Human Genome Research Institute. Available at: <https://www.genome.gov/genetics-glossary/Recombinant-DNA-Technology>.
- Wills, M. (2020). *Isinglass; or, The Many Miracles of Fish Glue*. [online] JSTOR Daily. Available at: <https://daily.jstor.org/isinglass-or-the-many-miracles-of-fish-glue/>.
- Wingfield, P.T. (2015). Overview of the Purification of Recombinant Proteins. *Current Protocols in Protein Science*, [online] 80(6), pp.6.1.1–6.1.35. doi:<https://doi.org/10.1002/0471140864.ps0601s80>.
- Wong, T. (2023). Fukushima: Anxiety and anger over Japan’s nuclear waste water plan. *BBC News*. [online] 5 Jul. Available at: <https://www.bbc.co.uk/news/world-asia-66106162>.
- Wu, J., Li, Z., Yuan, X., Wang, P., Liu, Y. and Wang, H. (2011). Extraction and isolation of type I, III and V collagens and their SDS-PAGE analyses. *Transactions of Tianjin University*, 17(2), pp.111–117. doi:<https://doi.org/10.1007/s12209-011-1543-2>.

Wu, X., Cai, L., Cao, A., Wang, Y., Li, T. and Li, J. (2015). Comparative study on acid-soluble and pepsin-soluble collagens from skin and swim bladder of grass carp (*Ctenopharyngodon idella*). *Journal of the Science of Food and Agriculture*, 96(3), pp.815–821. doi:<https://doi.org/10.1002/jsfa.7154>.

Wu, X., Zhang, Y. and Feng, X. (2023). The impact of Japanese nuclear wastewater discharge into the sea on the global economy: Input-output model approach. *Marine Pollution Bulletin*, [online] 192, p.115067. doi:<https://doi.org/10.1016/j.marpolbul.2023.115067>.

Xiong, X., Ghosh, R., Hiller, E., Drepper, F., Knapp, B., Brunner, H. and Rupp, S. (2009). A new procedure for rapid, high yield purification of Type I collagen for tissue engineering. *Process Biochemistry*, 44(11), pp.1200–1212. doi:<https://doi.org/10.1016/j.procbio.2009.06.010>.

Xu, C., Yu, Z., Inouye, M., Brodsky, B. and Mirochnitchenko, O. (2010). Expanding the Family of Collagen Proteins: Recombinant Bacterial Collagens of Varying Composition Form Triple-Helices of Similar Stability. *Biomacromolecules*, 11(2), pp.348–356. doi:<https://doi.org/10.1021/bm900894b>.

Yang, C., Hillas, P.J., Baez, J.A., Nokelainen, M., Balan, J., Tang, J., Spiro, R. and Polarek, J.W. (2004). The Application of Recombinant Human Collagen in Tissue Engineering. *BioDrugs*, [online] 18(2), pp.103–119. doi:<https://doi.org/10.2165/00063030-200418020-00004>.

Yang, M., Yang, Z., Liu, C., Lee, X. and Zhu, K. (2022). Characterization of the Complete Mitochondrial Genome of the Spotted Catfish *Arius maculatus* (Thunberg, 1792) and Its Phylogenetic Implications. *Genes*, [online] 13(11), p.2128. doi:<https://doi.org/10.3390/genes13112128>.

Yu, E.T., Liu, B.X., Wang, G., Yu, D., Xie, J., Xia, Y.X., Gong, W.X., Wang, H., Li, Z.B. and Wei, N. (2014a). Molecular cloning of type I collagen cDNA and nutritional regulation of type I collagen mRNA expression in grass carp. *Journal of Animal Physiology and Animal Nutrition*, 98(4), pp.755–765. doi:<https://doi.org/10.1111/jpn.12132>.

Yu, Z., An, B., Ramshaw, J.A.M. and Brodsky, B. (2014b). Bacterial collagen-like proteins that form triple-helical structures. *Journal of Structural Biology*, 186(3), pp.451–461. doi:<https://doi.org/10.1016/j.jsb.2014.01.003>.

Zdzieblik, D., Oesser, S., Baumstark, M.W., Gollhofer, A. and König, D. (2015). Collagen peptide supplementation in combination with resistance training improves body composition and increases muscle strength in elderly sarcopenic men: a randomised controlled trial. *British Journal of Nutrition*, 114(8), pp.1237–1245. doi:<https://doi.org/10.1017/s0007114515002810>.

Zhang, H., Wu, X., Quan, L. and Ao, Q. (2022a). Characteristics of Marine Biomaterials and Their Applications in Biomedicine. *Marine Drugs*, 20(6), p.372. doi:<https://doi.org/10.3390/md20060372>.

Zhang, J., Deng, Q., Liu, X., Wu, M.-H., Ma, Z., Shaw, P.-C., Zhang, Y. and Cao, H. (2022b). Molecular Identification of Commercial Fish Maws by DNA Sequencing of 16S rRNA and Cytochrome c Oxidase I Genes. *Journal of Food Protection*, 85(10), pp.1439–1445. doi:<https://doi.org/10.4315/jfp-22-121>.

Zielińska, S., Radkowski, P., Blendowska, A., Ludwig-Gałęzowska, A., Łoś, J.M. and Łoś, M. (2017). The choice of the DNA extraction method may influence the outcome of the soil microbial community structure analysis. *MicrobiologyOpen*, [online] 6(4), p.e00453. doi:<https://doi.org/10.1002/mbo3.453>.