UMT PRESS

Planetary Sustainability
Journal Homepage: https://journal.umt.edu.my/index.php/planetsust/
eISSN: 3009-0105
DOI: http://doi.org/10.46754/ps.2025.01.003

HARNESSING MARINE INVERTEBRATES: THE EXTRACTION TECHNIQUES OF COLLAGEN

NURUL IFFAH JUHARI[1], EMIENOUR MUZALINA MUSTAFA[2] AND NURUL ULFAH KARIM[3]*

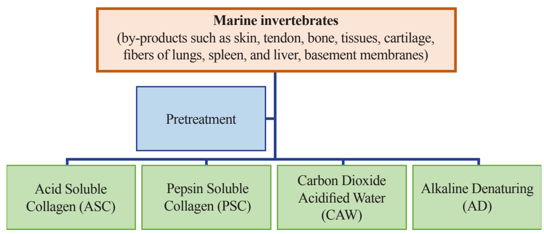
[1]Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, Malaysia. [2]Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia. [3]Higher Institution Centre of Excellence, Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.

**Corresponding author: ulfah@umt.edu.my*

HIGHLIGHTS

- Marine collagen plays an important role in functional foods and nutritional supplements.
- Marine collagen is characterised by a helical structure composed of amino acids like glycine, proline, and hydroxyproline.
- There are several marine collagen extraction techniques that are widely used, including acid soluble collagen, pepsin soluble collagen, carbon dioxide acidified water, and alkaline denaturing.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article History:
Received: 10 December 2024
Revised: 23 December 2024
Accepted: 25 December 2024
Published: 20 January 2025

Keywords:
Marine collagen, collagen extraction, marine invertebrates, crustaceans.

ABSTRACT

Marine resources offer a sustainable alternative to conventional protein and nutrient sources with marine collagen playing an important role in functional foods and nutritional supplements. Collagen from marine invertebrates is widely used in the food industry, pharmaceutical products, and biomedical applications. The aim of this study is to study the techniques used to extract collagen from various marine invertebrates. Marine collagen is a structural protein characterised by a helical structure composed of amino acids like glycine, proline, and hydroxyproline. Collagen comprises of 28 different types but only four types: Type I, II, III, and IV have always been studied. Type I is obtained from skin, tendon, and bone, meanwhile Type II is obtained from tissues of vitreous body, cartilage, and nucleus pulposus. Type III is obtained from the vessel walls and reticular fibres of lungs, spleen, and liver and Type IV is obtained from basement membranes. The extraction of marine collagen involves three stages: Pretreatment, extraction, and recovery. Several marine collagen extraction techniques are commonly used, including acid soluble collagen, pepsin soluble collagen, carbon dioxide acidified water, and alkaline denaturing procedures. Different extraction methods produce collagen yields with varying chemical compositions and characteristics. All of the extraction processes are ethical and environmentally friendly.

Introduction

Marine resources are a sustainable alternatives to conventional protein and nutrient sources. Marine collagen has established in functional foods and nutritional supplements that can contribute to global food security measures. Collagen peptides derived from marine invertebrates provide essential amino acids, boosting bone and joint health, as well as skin regeneration. Furthermore, marine collagen is an alternative source of conventional proteins that can address protein malnutrition and improve nutritional outcomes, especially in regions where animal-based protein sources are scarce or unsustainable. The use of marine invertebrates as a renewable resource also contributes significantly to the conservation of marine ecosystems. In addition, to the efficient use of renewable marine resources and by-products can reduce waste and minimise the environmental impact of acquiring these protein and nutrient foodstuffs, which supports sustainable food production models.

Marine collagen can be derived from the conversion of waste created by fishing industry activities, including fish markets and fish processing factories (Parisi *et al*., 2019). However, the waste produced including the skin, bones, fins, heads, viscera, and scales are still not begun properly disposed of and till date no relevant action has been taken to address this issue. A wide range of waste disposal methods have been undertaken but these methods all pollute the environment. The use of landfills and incinerators are both not viable options as they require high initial investments and are costly to run and maintain even afterwards (Ahmed *et al*., 2020). The collagen from bovine and porcine sources can pose a danger to human health by way of some transmissible diseases, like Foot-and-Mouth Disease (FMD) and Bovine Spongiform Encephalopathy (BSE). Therefore, marine collagen may provide consumers with a safer product option free if concern of such infections. (Ahmed *et al*., 2020). Transmissible diseases like FMD and BSE have also added to religious concerns over the safety of collagen derived from bovine and porcine sources.

Collagen is a large family of proteins that is comprised of three polypeptide chains, twisted into a triple-helix structure. Generally, collagen is found in the connective tissue, extracellular matrix, and building blocks of bones, skin, hair, ligaments, tendons, joints, and cartilage (Coppola *et al*., 2020). Some marine organisms are a good source of collagen. They are a well-known alternative to porcine and bovine collagen and have better properties, including a unique biocompatibility and little to no religious or ethical conflict regarding its use (Subhan *et al*., 2020). In recent years, the demand of marine collagen has increased, attracting the attention of researchers and industry players.

Collagen has a wide range of applications due to its excellent degradability and biocompatibility. It has been categorised into 28 different types (Meyer, 2019). However, only four types have been constantly researched, Type I, II, III, and IV. Type I is obtained in many tissues such as skin, tendon, and bone. Type II is obtained from the tissue of vitreous body, cartilage, and nucleus pulposus, while Type III is derived from the vessel walls and reticular fibres of lungs, spleen, and liver and Type IV is derived from basement membranes. The tissues of marine invertebrates (Cnidaria and Porifera) contain are composed of about 60% of essential Type IV, V, and II collagen (Avila *et al*., 2018). Collagen fibres have a complex triple helix structure with stable inter- and intra-molecular hydrogen bond crosslinks (Sionkowska *et al*., 2017). These bonds are challenging to be solubilised; therefore, specific methods are needed during the extraction process to improve the solubilisation of the collagen proteins and obtain a better extract.

Collagen from bovine and porcine sources can transmit diseases that have a detrimental effect on human health. Additionally, there are religious issues when dealing with collagen derived from bovine and porcine sources. As stated by Huang *et al*. (2016), Muslims and Jews are forbidden from eating pork, whereas Hindus do not consume beef. Muslims, Jews, and Hindus account for approximately 38.4% of the global population, which limits the viability of mammal-derived collagen (Regulations CE n. 999/2001 and UE n. 142/2011) (Coppola *et al*., 2020). Marine collagen offers a better and safer alternative product without any concern of such diseases' infections and has little to no religious or race-based cause for conflict.

The aim of this review is to identify the marine invertebrate species that are an abundant collagen source and to compare the most effective method or technique of collagen extraction from these marine species. This study provides a comprehensive data in developing and introducing effective methods of collagen extraction from marine invertebrate species and thus may reduce the environmental, ethical, and religious issues.

Marine Collagen Overview

Collagen is a structural protein in the extracellular matrix of skin, ligaments, bones, cartilage, and tendons of vertebrates and invertebrates (Jafari *et al*., 2020). Collagen molecules are characterised as a stiff three-strand helical structure arising from amino acids; glycine (Gly), proline (Pro), and hydroxyproline (Hyp). The most common amino acid sequence of the collagen is Gly-Pro-X and Gly-X-Hyp, where X can be any amino acid. Jafari *et al*. (2020) added that marine collagen has low molecular weight and small particle size that helps increase the absorption sate by up to 1.5 times and it has a better circulatory flow. Marine collagen can be categorised into two types: Depending on the source (invertebrates or vertebrates). Marine invertebrates have a limited supply of potential collagen sources (Tziveleka *et al*., 2017). Marine collagen has a lower denaturation temperature of between 25°C and 30°C for most fish species compared to mammalian collagen which have denaturation temperatures of between 39°C and 40°C. This is because they have lower amino acid concentrations (Pro and Hyp), making them a little difficult to handle because they tend to denature at the internal temperature of human beings.

Marine collagen has been used in many industries. The food industry has the greatest number of applications for collagen, representing 29.0% of the global collagen market (Pang, 2016). Marine collagen is a popular element in the formulation of nutritious meals. Type I collagen has been used in various applications, including as a food additive to improve colour, texture, flavour, and quality. In the cosmetics industry, Luca *et al*. (2020) stated that marine collagen was highlighted as a booster for skin elasticity, hydration, and to minimise wrinkles. Meanwhile, in biomedical and pharmaceutical applications, marine collagen is commonly used to make wound dressing, vitreous implants, and carriers for drugs, proteins, and gene delivery systems (Meyer, 2019). Some marine collagen containing live cells such as keratinocyte and fibroblast are capable of proliferating cytokines and growth factors. These biological molecules can provide for skin regeneration and wound healing in burn patients.

Collagen Extraction Techniques

Generally, collagen is isolated in a three-step process: Pretreatment, extraction, and recovery. Pretreatment involves a cleaning process, which will remove contaminants, separate raw materials into different groups (bone, skin, scales, etc.), and size reduction (cutting and mincing) (Muthukumar *et al*., 2018). In the extraction phase, various conventional extraction methods are used to extract collagen. The yield, chemical composition, and characteristics of the extracted collagen vary between method. Collagen is precipitated during the recovery process, usually by adding natrium chloride (NaCl) to the final concentration. The precipitate was collected by centrifugation before being dissolved in acetic acid and freeze-dried (Fassini *et al*., 2020).

Acid Soluble Collagen (ASC)

Acid Soluble Collagen (ASC) is the among common treatment used to extract and purify collagen in various industries. This extraction technique mainly relies on acid hydrolysis. The extraction can utilise either organic acids (citric acid, acetic acid, lactic acid) or inorganic acids (phosphoric acid, hydrochloric acid), since they able to solubilise most of the collagen macromolecules into a single tropocollagen chains (Schmidt *et al*., 2016). Fassini *et al*. (2020) stated that 0.5 M acetic acid solution is the most used acid for this collagen extraction technique. The acidic solution enhances the repulsion between tropocollagen molecules, which will assist in collagen molecule solubilisation (Ahmed *et al*., 2020). The acidic extraction solution usually consists of a concentration ranging between 0.5 to 1 M, allowing for the solubilisation of intra- and inter-molecular crosslinks without altering the structure of the collagen chains. Figure 1 shows the summary of ASC extraction technique.

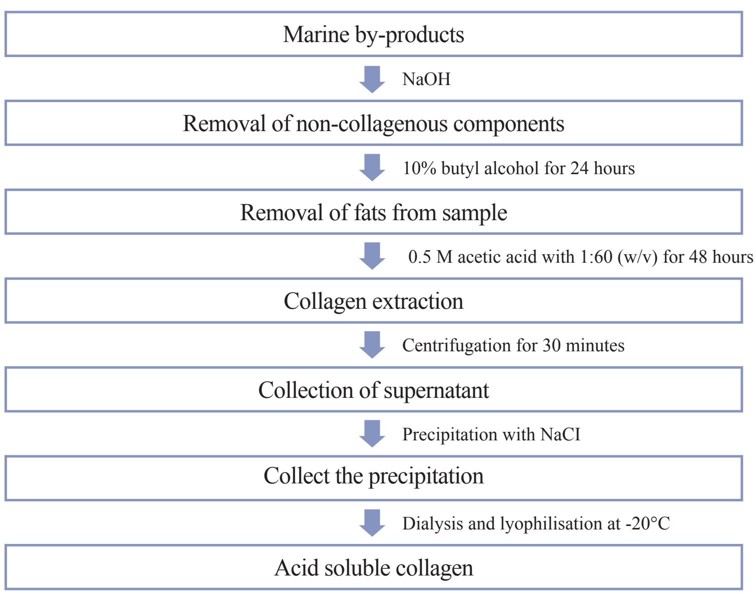


Figure 1: Summary for Acid Soluble Collagen (ASC) extraction technique

Source: Fassini *et al*. (2020)

Pepsin Soluble Collagen (PSC)

Pepsin Soluble Collagen (PSC) extraction methods is identical to that of the ASC, with the exception being that the samples are extracted using 0.5 M acetic acid containing 10% (w/w) pepsin and dialysed against 0.02 M Na_2HPO_4 until the pH was more than 8.0. It functions to inactivate the pepsin molecule before further dialysing against 0.1 M acetic acid. According to Schmidt *et al*. (2016), PSC also uses enzymatic hydrolysis, which improves the reaction selectivity and is less damaging to the collagen protein. Pepsin enzymes that are added to the acidic medium have a stronger crosslinking linkage compared to the telopeptide sections. The telopeptides will be cleaved by the enzymes while the helical area remains intact. Figure 2 is a summary of the PSC extraction method.

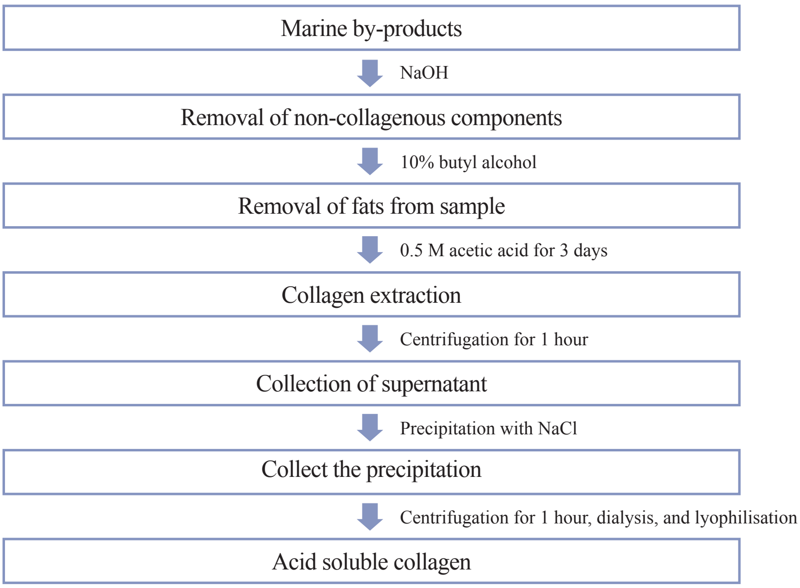


Figure 2: Summary for Pepsin Soluble Collagen (PSC) extraction technique

Source: Schmidt *et al.* (2016)

Carbon Dioxide Acidified Water (CAW)

Carbon Dioxide Acidified Water (CAW) method utilise carbon dioxide and acidified water to extract collagen from its source. This method is considered as a “greener” method as it does not use any contaminants or modified chemicals for the extraction (Silva *et al*., 2016). The use of carbon dioxide and water should not leave any substances behind that could harm human beings or the environment. The use of high-pressure processing will speed up the process, reducing the time, and cost involved. Figure 3 is a summary of the CAW extraction method.

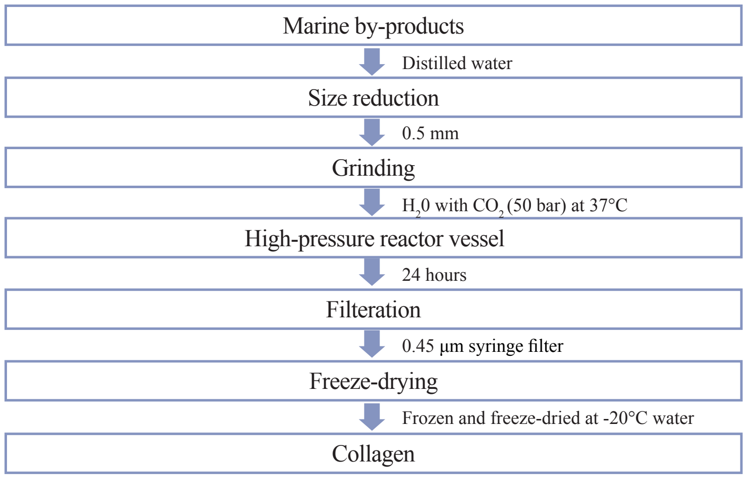


Figure 3: Summary for Carbon Dioxide Acidified Water (CAW) extraction technique

Source: Silva *et al.* (2016)

Alkaline Denaturing (AD)

An Alkaline Denaturing (AD) treatment is a good technique for collagen extraction as it produces a high recovery yield even from old sources. Alkali is usually used in the pretreatment process to help with the solubilisation. It is particularly effective for collagen extraction from a source with a thick, hard protective layer (Tziveleka *et al*., 2017). The duration of the treatment depends on the source, which might take a few days and even week. NaOH is one of the more widely used alkali for this purpose as it swells the skin helping the alkali diffuse into the source. The alkali will hydrolyse the undesired non-collagenous proteins, pigments, and other organic materials. It also will not have any effect on the thermal stability and triple helical composition of the collagen. Figure 4 illustrates the Alkaline Denaturing (AD) process of collagen extraction.

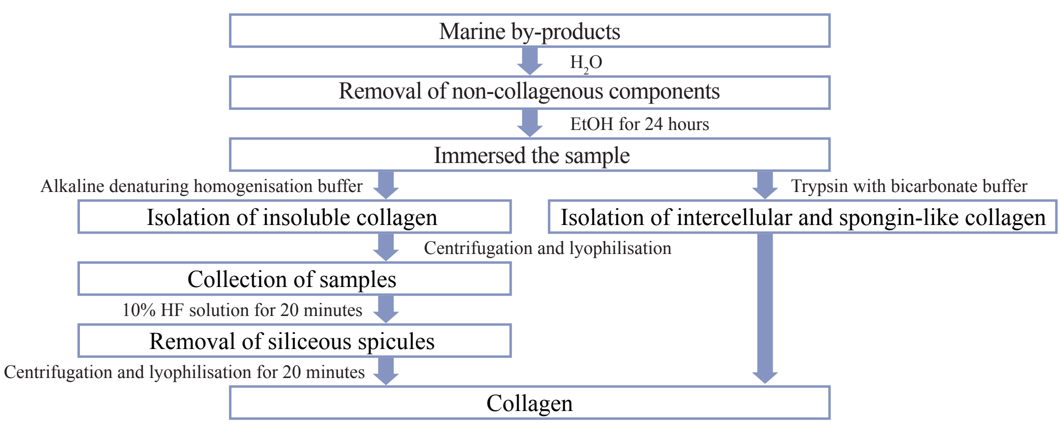


Figure 4: Summary of Alkaline Denaturing (AD) collagen extraction

Source: Tziveleka *et al.* (2017)

Collagen Extraction from Marine Invertebrate

Porifera (*Marine Sponge)*

Marine sponges belong to the phylum of *Porifera*, comprise of a mass of cells including a porous skeleton made of organic (collagen fibres and/or sponging, class of Demospongiae) and inorganic (spicules) elements. Among multicellular animals (Metazoa), they are both the most primal. In sponges, fibrillar collagen makes up most of the organic material. Non-fibrillar collagen (called spongin) is a short-chain molecule encoded in sponges (Berillis, 2015). Sponges' collagen (Demospongiae) is solely the intercellular organic structure and accounts for approximately 10% of the overall organic matter (Tziveleka *et al*., 2017). The extraction procedure for sponges is for Freeze-dried (FD), Carbon Dioxide Acidified Water (CAW), and Alkaline Denaturing (AD) as illustrated in Table 1.

Table 1: Source, yield of collagen, and extraction method from *Porifera* species

Sponge Species	Common Name	Tissue	Yield (%)	Method	References
Chondrilla nucula	Chicken liver sponge	Whole	82.0	CAW	Barros *et al*. (2015)
Chondrosia reniformis	Kidney sponge	Whole	35.5	FD	Gökalp *et al*. (2020)
Chondrosia reniformis	Kidney sponge	Whole	30.4	CAW	Silva *et al*. (2016)
Axinella cannabina	Orange candlestick	Whole	12.6	AD	Tziveleka *et al*. (2017)
Suberites carnosus	Fleshy horny sponge	Whole	5.0	AD	Tziveleka *et al*. (2017)
Chondrilla caribensis	Chicken liver sponge	Whole	1.3	FD	Araújo *et al*. (2021)
Aplysina fulva	Scattered pore	Whole	1.1	FD	Araújo *et al*. (2021)

Note: FD = Freeze-dried, CAW = Carbon Dioxide Acidified Water, and AD = Alkaline Denaturing.

Cnidaria (*Jellyfish)*

Jellyfish is a prominent source of marine collagen as their collagen content can exceed 60% (Avila *et al*., 2018). Jellyfish oral arms are separated from the umbrella and divided into mesoglea, exumbrella, and subumbrella. Mesoglea have been found to have the most collagen as compared to the exumbrella and subumbrella (Khong *et al*., 2018). Recently, researchers reported some jellyfish extraction from Type I tissues considerably comparable to the human class. Subhan *et al*. (2015) found that the jellyfish collagen is a non-toxic source that provides numerous antioxidants and had higher cell viability (fibroblastic, epithelial, osteoblastic, and fibrosarcoma). As shown in Table 2, jellyfish collagen extraction methods are mainly ASC- or PSC-based. The PSC method presented has the potential to provide a significantly higher collagen yield.

Table 2: Source, yield of collagen, and extraction method from *Cnidaria* species

Jellyfish Species	Common Name	Tissue	Yield (%)	Method	References
Rhopilema esculentum	Flame jellyfish	Mesoglea	0.28	PSC	Cheng *et al*. (2017)
Acromitus hardenbergi	River jellyfish (marine)	Oral arms	0.39	PSC	Khong *et al*. (2018)
Catostylus mosaicus	Blue blubber jellyfish	Umbrella	14.58	PSC	Hajiani and Osfouri (2021)
Catostylus mosaicus	Blue blubber jellyfish	Oral arms	12.8	PSC	Hajiani and Osfouri (2021)
Rhopilema esculentum	Edible flame jellyfish	Mesoglea	0.28	PSC	Cheng *et al*. (2017)
Rhopilema esculentum	Edible flame jellyfish	Mesoglea	0.12	ASC	Cheng *et al*. (2017)
Chrysaora sp.	Ribbon jellyfish	Umbrella	9.0-19.0	PSC	Barzideh *et al*. (2014)

Note: ASC = Acid Soluble Collagen and PSC = Pepsin Soluble Collagen.

Echinodermata

Phylum *Echinodermata* usually recognisable by their radially symmetrical body composition, that usually consists of five-pointed limbs. Another distinct characteristic is their watery vascular system. These phyla mainly consist of sea star (starfish), sea urchin, brittle stars, and sea cucumbers. *Asterias pectinifera*, a species of hazardous marine starfish has currently been recognised as an alternative for a water soluble, low molecular weight, and is an eco-friendly source of collagen peptides (Han *et al*., 2021). Most organisms in this phylum were inedible and some of them are invasive and a hazard to their marine ecosystems (*Acanthaster planci*, crown-of-thorn starfish), making them a sustainable alternative collagen source (Tan *et al*., 2013). However, sea cucumbers can produce more collagen than sea stars, as can be seen in Table 3.

Table 3: Source, yield of collagen, and extraction method from *Echinodermata* species

Species	Common Name	Tissue	Yield (%)	Method	References
Holothuria scabra	Sand sea cucumber	Flesh	6.0	ACS	Syahputra *et al*. (2021)
Asterias pectinifera	Blue bat star (starfish)	Full body	3.8	PSC	Han *et al*. (2021)
Stichopus japonicus	Japanese spiky sea cucumber	Body wall	70.0	PCS	Saito *et al*. (2002)
Holothuria scabra	Sand sea cucumber	Flesh	20.76	ACS	Desmelati *et al*. (2020)
Holothuria cinerascens	Black sea cucumber	Full body	72.22	PSC	Li *et al*. (2020)
Acanthaster planci	Crown-of-thorn starfish	Body wall	2.29	PSC	Tan *et al*. (2013)
Asterias amurensis	Northern pacific sea-star	Full body	3.0	PSC	Li *et al*. (2023)
Paracentrotus lividus	Purple sea urchin	Peristomia membrane	4.93	FD	Ferrario *et al*. (2020)

Note: ASC = Acid Soluble Collagen, PSC = Pepsin Soluble Collagen, and FD = Freeze-dried.

Crustacea

Crustaceans are subphylum under a larger phylum group of *Arthropoda*. This group usually consist of prawn, shrimp, lobster, crayfish, and crab. Crustaceans are different from other class of arthropods (insects, myriapods, and chelicerates) by possessing two-parted (biramous) limbs and by growing from larval forms (in their lifecycle). Crustaceans have hard exoskeletons, which are commonly called shells. Species from this subphylum are not a popular source for the extraction of collagen (Hiransuchalert *et al*., 2021) and not many researchers have focussed their research on it. The focus was more towards extracting the chitosan, gelatine, and chitin from the shell (Ait Boulahsen *et al*., 2018). The collagen content in this subphylum species was very low and was not cost-effective. Only a handful of researchers studied collagen extraction from crustacean sources, as shown in Table 4.

Table 4: Source, yield of collagen, and extraction method from *Crustacea* species

Species	Common Name	Tissue	Yield (%)	Method	References
Miyakella nepa	Three-banded mantis shrimp	Muscle	0.5	PCS	Hiransuchalert *et al*. (2021)
Oratosquilla nepa	Mantis shrimp	Muscle	42.0	ASC	Preetha Mini Jose *et al*. (2014)

Note: ASC = Acid Soluble Collagen and PSC = Pepsin Soluble Collagen.

Mollusca

Mollusca is one of the largest phyla in animal kingdom, which consist of over 85,000 species. They can be recognised by its unsegmented body, mostly been protected by calcareous shell, the body contains a cavity, and their head comprised of tentacles with a compound eye. *Mollusca* can be found in both saltwater (marine) and freshwater with rather similar characteristics. The phylum mainly consists of snails, squid, cuttlefish, octopus, and bivalves. Bivalves and Cephalopods (squid, cuttlefish, etc.) have been consumed by humans throughout history; however, not many know that they are a good source of bioactive collagen (Agbaje *et al*., 2021). Various researchers had studied the collagen extracted from mollusc waste due to their sustainable nature. Table 5 shows the source, collagen yield, and collagen extraction methods for mollusc.

Table 5: Source, yield of collagen, and extraction method from *Mollusca* species

Species	Common Name	Tissue	Yield (%)	Method	References
Callista disrupta	Venus clams	Shells	1.38	ASC	Agbaje *et al*. (2021)
Mactra chinensis	Sunray surf clam	Edible parts	30.5-39.0	ASC	Tabakaeva *et al*. (2018)
Sepia lycidas	Cuttlefish	Outer skin waste	35.0	PSC	Nagai *et al*. (2001)
Kondakovia longimana	Antarctic squid	Skin	1.18	PSC	Coelho *et al*. (2017)
Dosidicus gigas	Humbolt squid	Skin	32.86	PSC	Cao *et al*. (2022)

Note: ASC = Acid Soluble Collagen and PSC = Pepsin Soluble Collagen.

Conclusions

Collagen derived from marine invertebrates have proven to be a good and more sustainable alternative than mammal-derived collagen. Additionally, there are little to no religious issues with its use, which make it more acceptable to a wider range of people. Some marine invertebrates proved to be very efficient sources of collagen, with the percentage yield reaching as high as 82%. The collagen industry is expected to continue to grow in the coming years, opening up new exciting and challenging opportunities in this research area.

Funding

This research was funded by The Ministry of Higher Education Malaysia (MOHE) under HICOE Research Grant Scheme (HICoE AKUATROP/2025).

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Acknowledgements

The authors would like to thank Ministry of Higher Education (MOHE), Malaysia and Higher Institution of Excellence (HICoE), Institute of Tropical Aquaculture and Fisheries (AKUATROP), Universiti Malaysia Terengganu for laboratory facilities support.

Conflicts of Interest Statement

The authors declare no conflict of interest.

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