ORIGINAL ARTICLE

Extraction of Fish Collagen Peptides from Fish Waste through Fermentation using Lactobacillus Bacteria

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ABSTRACT

Objective: To introduce a novel technique for extracting fish collagen peptides from fish waste. It could be a cheap and safe technique for obtaining peptides of fish as well as reducing the fish waste environmental load.

Study Design: A comparative study.

Place and Duration of Study: The study was conducted at Department of Microbiology, Hazara University Mansehra, Khyber Pakhtunkhwa and National University of Medical Sciences, Rawalpindi, Pakistan from August 2020 to July 2022.

Methods: For the extraction of the fish collagen peptides, lactobacillus bacteria fermentation was being performed, in which the bacteria from yogurt and dough were added to fish samples and incubated for one month at 30°C. Lactobacillus bacteria were diluted with serial dilution for colonies isolation and biochemical characterization. Fish waste was cut in small pieces and put in a bottle containing distilled water and lactobacillus bacteria species and kept at 30°C for one month. The Hestrin–Schramm media (HS) was added in small amount as a starter for initial growth of bacteria. The pH was analyzed after every 7 days and 5–10 ml sample was collected from each labelled flask. The collected samples were precipitated using Trichloroacetic acid (TCA) and analyzed on SDS-PAGE.

Results: Gel analysis revealed different size of fish collagen with higher concentration include the pattern for α1 and α2 chains with a molecular weight of 145 kDa and 132 kDa, respectively. The biological activity of extracted fish collagen was determined by using HaCaT cells proliferation analysis.

Conclusion: The current study concluded that fish waste could be converted to biofunctional collagen using nonpathogenic strains of lactobacillus bacteria for various biomedical applications.

Keywords: *Extraction, Fish Waste, Fish Collagen Peptides, Lactobacillus.*

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Introduction

Proteins are the structural and functional units of animal body and among them collagen provide the most abundant part of the total protein, which constitute 25-35% of all the protein in an animal's body. The connective tissues of bone and skin of the animal body are mainly comprised of collagen protein.[1,2] The scientific innovation investigated the applications of collagen protein in diverse sectors like food, film, biomedical, pharmaceutical, cosmetic and leather industries.[3,4]

Plants, animals, and fish collagen are extracted at

industrial level. However, compared to protein from plant and animal sources, fish collagen protein has a number of advantages.[5]

The fish collagen is regarded as a more useful source of collagen with applications in various health and biomedical industries. When compared to collagen protein from terrestrial animals, which may carry various transmissible infectious diseases, fish collagen protein can be considered as a healthier alternative. Hence, industrial waste from fish has the potential to be an alternative source for producing collagen protein.[6]

Fish collagen is a source of protein that is soluble in water, and because of its unique properties, it is used in a variety of industries such as pharmaceuticals, food and cosmetics due to its special features.[7] There are slight differences in the physical attributes and chemical nature of fish collagen compared to animal collagen, but overall, their molecular functions are similar.[5,8,9]

Due to its diverse properties, collagen is important for industrial applications and is one of the most important biomaterials.[9] Collagen and gelatin are one of the most sought-after products in the food industry, as they are used for gel formation, stabilization of emulsions and have high water absorption capacity.[8] Similarly, collagen has several applications as a potential biomaterial, including drug delivery, gene delivery, regeneration of human blood vessels, skin and ligaments, as a therapeutic protein and for wound healing.[10]

For microbiologist, the term 'fermentation' refers to any process utilized for production of a variety of products through the mass cultivation of microbes. From the perspective of a biochemist, fermentation is a biochemical process where organic compounds react (as both electron donors and acceptor) to produce energy. Fermentation technology offers one of the best opportunities for food innovation, such as recent research on innovative foods that highlights its fermentative impact on food safety, high energy content and long-term shelf life.[11]

The microbes ferment in the absence of oxygen to produce energy.[12] The fermented food is processed and modified once selected microorganisms and specific enzymes show their activity leading to the required biochemical changes.[13] A microbial ecosystem that lives on the countertop and is kept in food sales worldwide can be used to describe the relationship related to safe food. Mold, bacteria, and yeast are the main components of the fermentation ecology, and the microbes that live in food containers protect the food they ingest.[14]

Market demand and potential applications of collagen are prompting researchers to explore potential sources for collagen production. Fish waste (skin, scales, and bones) are considered the most important characteristics for mammalian collagen among all available potential sources for collagen production. Therefore, due to the current scientific innovation and potential market demand, the industry has become aware of marine collagen.[15]

In this context, two main known methods are used to obtain collagen from fish waste, namely chemical and enzymatic. However, these methods are expensive or may be harmful due to the use of toxic chemicals for collagen extraction.[16]

Keeping all this in view, objective of present study is to discover those biological techniques that are inexpensive and safe for collagen extraction from fish wastes. Therefore, alternative method of extraction could be applied to safeguard our environment from waste pollution.

Methods

Collection of Specimens and Storage

The current study was conducted at Department of Microbiology, Hazara University Mansehra, Khyber Pakhtunkhwa and National University of Medical Sciences, Rawalpindi, Pakistan from August 2020 to July 2022. The fish waste was collected from the local market followed by three times washing at room temperature using distilled water and stored at -20°C until use. The required Hestrin–Schramm (HS) media was prepared by adding 20g of D-glucose, 5g peptone, 5g yeast extract, 2.7 g Na2HPO4, and 1.15 g of citric acid in 1000 ml of distilled water. All the above chemicals were purchased from Gibco (Gibco Life Technologies, Grand Island, NY, USA). The pH of the medium was adjusted 6.0 and was autoclaved at 121°C for 15 minutes.

Preparation of Agar Plates

The agar (Sigma Aldrich, St. Louis, USA) plates were prepared using 6g of agar in 100 ml HS media. Heat plate stirrer was used to warm up media and the agar

was melted, which was cooled down for easy handling. Media was then poured in petri plates (15–20 ml), cooled, and stored at 4°C until use.

Culturing on Petri Plates

The dry dough and yogurt were collected from the local market followed by dilution using distilled water. With the help of sterile swab, yogurt and diluted dough were streaked on various petri plates having HS agar media in them. Later, these petri plates were incubated at 37°C temperature for 24 hours. Different colonies were selected and transferred to broth HS culture media and culture for 24 hours at 37°C. For later use and experimentation, stock was reserved at 4°C.

Fish Specimen Processing

Before fish waste fermentation, the fish were cut into small pieces of 1-2 cm and transferred to a 500 ml flask. A total of 10 different fermentation conditions were followed including a control medium and HS culture media with fish waste and isolated bacteria for fermentation. Flask 1 contained distilled water with fish waste, flask 2 contained yogurt with fish waste, flask 3 contain dough with fish waste and the last flask 4 contain HS culture media with fish waste only, respectively. The required experimental flasks were placed at 37°C temperature inside incubator. Supernatant samples were taken weekly from the incubated flasks to store at -80°C before collagen protein analysis. Samples were collected at weeks 1, 2, and 4 for collagen purification and SDS gel electrophoresis.

TCA Precipitation

The isolated supernatant samples of all fermentation condition at day 0, 7, 14, 21 and day 28 were proceed for TCA (Sigma Aldrich (St. Louis, USA) precipitation. For TCA precipitation, all samples were centrifuged at 4°C and 4500 rpm for 10 minutes. After completion of ultracentrifugation, only 3 ml of the supernatant was transferred to another 5 ml tube. Samples were centrifuged again at 2000 rpm for 5 minutes at 4°C, only 900 µl of the supernatant was transferred in triplicate to fresh 1.5 ml Eppendorf tubes and 100 µl of TCA was added to each tube with constant mixing. All samples were cooled to -20°C for 20 minutes and then centrifuged on microspin at 12,000 rpm for 10 minutes. The centrifuged tubes were slowly turned upside down to remove the supernatant, and 1 ml of 80% cold acetone was added to each tube, followed by another 3-minute centrifugation step at 10,000 rpm. After repeating the above step three times, the tubes were turned upside down and allowed to dry completely for 5 to 10 minutes. After drying, the remaining protein was dissolved in 100 µl of nuclease-free water (NFW) and stored at -20°C for further processing.

Gel preparation and Electrophoresis

Gel electrophoresis was performed using 6% separating and 5% stacking SDS-PAGE (sodium-dodecyl sulfate polyacrylamide gel electrophoresis). The 10 ml separating gel composition was 30% acrylamide mix (2 ml), 1.5 M Tris, 2.5 ml (pH 8.8), 10% SDS (0.1 ml), 10% ammonium persulfate 0.1 ml, TEMED 0.008 ml and distilled water 5.3 ml. Composition of separation SDS gel includes 0.83ml of 30% Acrylamide mix, 1.0 M Tris (pH 6.8) in quantity of 0.63 ml, 0.05 ml of 10% SDS, 0.05 ml of 10% Ammonium Persulfate, TEMED and distilled water in quantity of 0.005 ml and 3.4 ml, respectively. All the chemical required for SDS PAGE preparation were purchased from Sigma Aldrich (St. Louis, USA).

The fermented fish waste samples precipitated with TCA were analyzed using SDS-PAGE after adding 100 µl of SDS buffer (denaturing buffer) to each sample and denaturing it in a water bath at a temperature of 95°C for 5 minutes. Samples and protein marker were loaded at 10 µl each to SDS-PAGE. The loaded gel was run at a current of 100-200 volts in glycine buffer for 25 minutes. Coomassie Brilliant Blue G-250 (Sigma Aldrich St. Louis, USA) was used to stain the gel in a shaking incubator for 24 hours at 25°C. Collagen protein bands were decolorized after washing with acetic acid and methanol, and then photographed to visualize these bands.

Cell viability assay

HaCaT cells (obtain from American Type Culture Collection (ATCC) (Rockville, MD, USA) were seeded on both pure BC and BC-FCP composites for 24 to 72 h. Cell viability was analyzed through colorimetric WST-1 conversion assay (EZ-Cytox assay kit, Daeil Lab Service, Seoul, Korea) as per the given guidelines.

Statistical analysis

Experiments were performed in triplicate. We presented the data as mean standard deviation (SD).

The student's t-test was applied to calculate variances between groups and statistically important probability values were those which were less than 0.05.

Results

Lactobacillus isolation & identification

The results revealed the successful isolation of Lactobacillus from yogurt and dough by serial dilution and culture on agar plates (Figure 1). In this study, several tests were performed to identify Lactobacillus before the fermentation experiments (data not shown). The results showed the isolation of ten different Lactobacillus strains identified by the shape and size of the bacterial colonies on the agar plates. Subsequently, these bacterial strains were selected for fish waste fermentation by various biochemical tests (data not shown) and cultured in broth media prior to fermentation.

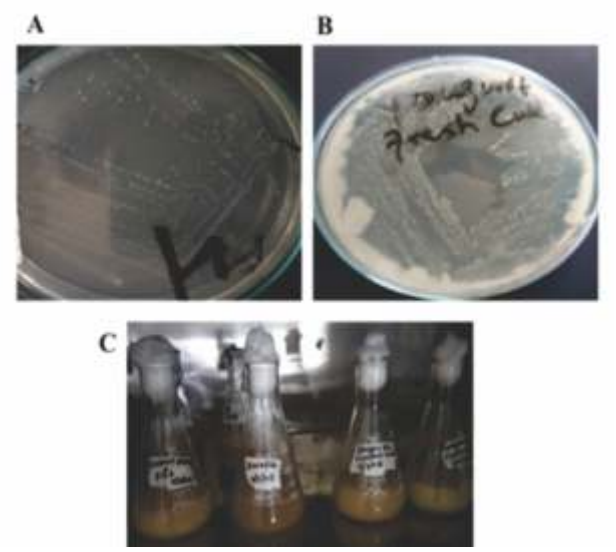

Fig 1: Bacterial culture of dough and yogurt (Fig A and B) by serial dilution using HS media and the fish waste collagen extraction through Lactobacillus bacteria fermentation (Fig C)

SDS-PAGE analysis

Results for SDS-PAGE showed that the collagen protein had doublet pattern for α1 and α2 chains with a molecular size of 145 kDa and 132 kDa (Yogurt + fish week 2 in figure shows clear identification), respectively (Figure 2).

The concentration of both α1 and α2 collagen protein are comparable on gel analysis. Fish waste fermentation was optimized at different temperatures from 30°C to 37°C. The current results show that 36°C and 37°C have a slightly better fermentation rate than lower temperatures. The pH concentration plays a very important role in the fermentation conditions, so in the present study, the pH was examined for all samples on a weekly basis and a slight increase in pH was observed with increasing incubation time (data not shown).

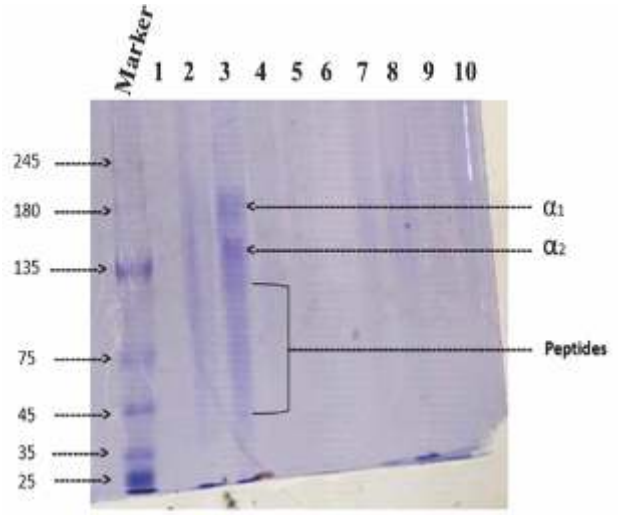

Fig 2: SDS-PAGE analysis, for separation and identification of fish waste lactobacillus fermented products of fish collagen. The figure labelled as (1) Distilled water + fish, (2) Yogurt + fish week 1 (3) Yogurt + fish week 2 (4) Yogurt + fish week 4 (5) Dough + fish week 1 (6) Dough + fish week 2 (7) Dough + fish week 4 (8,9,10) HS culture media + fish

Cellular cytotoxicity

Fermented fish collagen peptides and full-size mixture were examined for cellular cytotoxicity and proliferative effect using HaCaT cells. The results of cell proliferation showed that the collagen isolated from fish collagen fermentation was non-toxic and stimulated the proliferation of HaCaT cells compared to the control group (Figure 3).

Discussion

Although enzyme extraction is also used for fish collagen peptides and is considered more nutritious, most collagen used industrially is obtained by the chemical extraction method, so an alternative method of collagen extraction is still required.[17] Moreover, the enzymatic method, although expensive, leads to the production of less waste in a short time interval. The pretreatment step of using

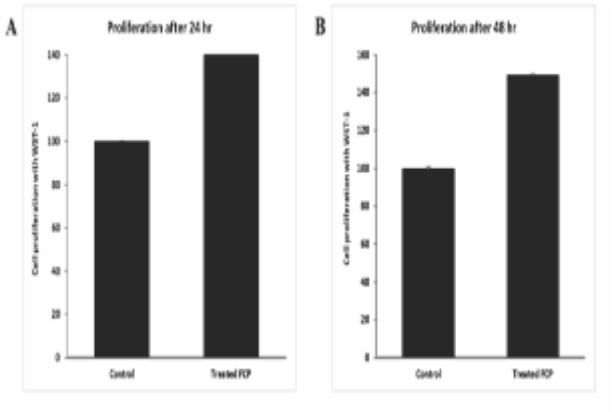

Fig 3: The human skin (HaCaT cells) proliferation effect. The human skin keratinocytes were culture in a 96-well plate with 1x104 cell per well. The cells were treated with FFCP at the rate of 0.5 %/ml for 24 (A) and 48 hr (B) respectively

an acidic or basic process is part of the chemical procedure before the collagen extraction process, depending in the origin of the raw material. Pretreatment methods can be used to remove non-collagenous components and to increase yields.[18] Collagen extraction methods are commonly employed using neutral saline solutions, acidic solutions, and basic solutions supplemented with enzymes.[19] Extensive efforts have been made to produce collagen with superior purity, high yield, preserved structural integrity, and special properties such as gelation, water retention, and thermal stability.[20]

We have selected lactobacillus bacteria for fish waste fermentation after taking numerous factors into consideration. Such as, researchers reported that fish peptone provide a better growing environment for some bacteria as compared to the culture median De Man, Rogosa and Sharpe agar (MRS). According to Safari et al., 2012, fish peptones are promising ingredients of industrial media for *Lactobacillus plantarum* and other food bacteria, because not only do they provide excellent growth support, but also because fish are not associated with meat-related diseases such as bovine spongiform encephalopathy (BSE).[21,22]

Another advantage of utilizing various strains of the Lactobacillus genus is their ability to produce different forms of lactic acid. Certain strains such as lactococcus, enterococcus, Aerococcus, Tetragenococcus, Vagococcus, Camobacterium, and Pisciglobus are known to produce l-lactic acid. On the other hand, specific lactobacillus strains and Leuconostoc are capable of producing d-lactic acid, while several other lactobacilli, pedicoccus, and Weissella strains have the capacity to produce dl-lactic acid.[23]

The SDS gel analysis showed a well-recognizable pattern of collagen type-1 peptides, and alpha-1 and alpha-2 bands can be observed in the majority of species.[24] Although the full size of collagen type-1 cannot be observed on the SDS gel. Additionally, the results of the SDS gel analysis clearly showed smaller collagen peptides. It was possible to clearly identify the two bands with alpha 1 and beta dimmer (between 135 and 180 kDa).[25] According to reports, fish collagen has no risk of becoming infected with a virus like BSE, however collagen from porcine skin BSE transmission chances.[26]

The biological activities of the fermented fish collagen peptides were also tested on HaCaT cells (human skin keratinocytes), with a similar result to other animal and fish collagen sources. The biological function studies showed that the fish collagen peptides caused inhibition of IL -6, IL -8 and TNF-α in biologically or chemically induced cells.[9,27] Previously, we determined that fish collagen protects skin cells against the cytotoxicity of heavy metals and biological inflammation in addition to promoting skin cell proliferation.[9]

Hence, fish collagen has emerged as a highly sought-after subject of scientific research due to its notable safety profile and potent biological properties. These properties include antioxidant and anti-tumor effects,[28] anti-hypertensive properties, neuroprotective capabilities,[29] anti-skin aging and promotion of epiphyseal growth,[30] as well as wound healing and the ability to enhance osteogenic and endothelial differentiation in rat bone marrow mesenchymal stem cells.[12]

Conclusion

Collagen extracted by different methods from different fish species studied has almost similar chemical properties. Fish waste provides a huge amount of collagen extraction sources for the future that mostly include the skin, bone, scale. Therefore, new techniques and tools are urgently needed to

obtain an alternative source of collagen from discarded fish waste. It is concluded that the current approach may be a novel method for extracting collagen from the fish skin, meat or other soft tissues using fermentation bacteria. The current method will provide low-cost, safe and fast collagen products especially from fish waste and reduce environmental pollution.

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Authors Contribution

SK: Data analysis, results and interpretation, manuscript writing and proof reading
IT: Data analysis, results and interpretation, manuscript writing and proof reading
MI: Data collection, data analysis, results and interpretation
SR: Manuscript writing and proof reading
RFS: Manuscript writing and proof reading
SS: Manuscript writing and proof reading
NAL: Manuscript writing and proof reading
LA: Manuscript writing and proof reading
FS: Idea conception, study designing, data analysis, results and interpretation, manuscript writing and proof reading

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