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Production and Application of Microbial Collagenase

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ABSTRACT

Enzymes known as collagenases are responsible for releasing collagen's peptide linkages. Nowadays, microbial collagenolytic proteases are receiving a lot of attention due to their better productivity and lower resource needs. The importance of proteases with collagenolytic activity stems from both their biological and industrial applications. They play a significant part in the recycling of collagenous waste on a global scale. Enzymes that break collagen's peptide bonds and trigger chemical reactions are called collagenases. Recent knowledge on collagen and collagenolytic proteases is provided in this review. In addition, the current study also discusses the most recent state of collagenases, including current methods and advanced technologies for microbial collagenases' separation, structure, production optimization, characterization, and applications.

Keywords: collagen, collagenase, microbial collagenase, application and enzymes.

1. Introduction

1.1. Collagen

Collagen serves as the body's main structural protein in both human and animal bodies. Collagens come in several varieties (types I, II, III, and IV). The most prevalent protein in bone and soft connective tissue is collagen type I. The major fibrillar collagen types supply a large portion of the structural framework for bone, cartilage, and soft connective tissues (Holmbeck & Birkedal-Hansen, 2013). Collagen's unusual structure contributes to its fibrous nature, which makes it exceptionally challenging to break down. With 30% of all proteins in an animal's body being collagen, it is the most common protein in mammals (Suzuki *et al.,* 2006; Pati *et al.,* 2010) .

The same macromolecule exists in diverse forms as collagen and gelatin. Collagen is partially hydrolyzed to provide gelatin, a soluble protein. Collagen and gelatine have been getting a lot of attention lately, especially in the food, beauty, medical, and cell culture industries. Most of the collagen and gelatine used in these industries comes from mammals like (cows and pigs), however in recent years, attention has been focused on the manufacture of collagen and gelatin from fish waste (Bhagwat & Dandge, 2018) .Collagen is the most common protein in the extracellular matrix of animals. It's a triple helical molecule that has three chains that mostly repeat each other as shown in (Figure 1). Sequences of the amino acid glycine, where the amino acids x and y are usually proline and hyxyproline (Kumar & Rani, 2017; Rani & Kumar, 2016).Because collagen is so complex and has a triple helix structure, it is highly resistant to most proteases. Most proteolytic enzymes, such as those in the proteinase family, can quickly cleave it.

Proteinases, such as those in the metalloproteinases class of proteolytic enzymes, can break down the extracellular matrix of collagen. There are at least three collagenases in this family of proteinases that can break down natural fibrillar collagen. Collagenases are the starting enzymes required for normal connective tissue turnover. In general, enzymes that may break down the polypeptide backbone are referred to as collagenases. They are separated into two categories that perform various physiological tasks. Serine collagenases are likely engaged in a number of biological processes, including the synthesis of hormones and pharmacologically active peptides. These

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processes include fertilization, complement activation, fibrinolysis, blood clotting, and protein digestion (Neurath, 1981; Bond & Van Wart, 1984; Shingleton, 1996).

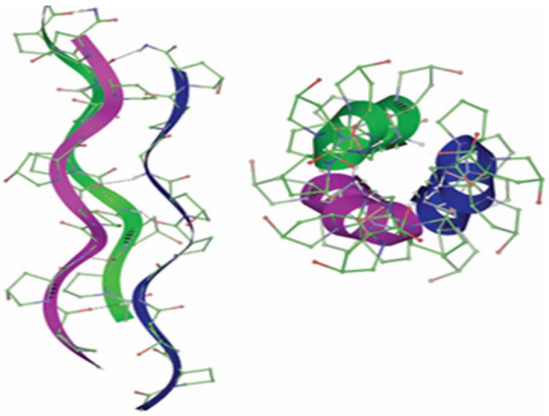

Fig. 1: Ball and stick diagrams of collagen triple helical structure for collagen (Bhattacharjee & Bansal, 2005) .

2. Collagenase History

Collagenase enzyme able to break down natural collagen in certain environments (temperature, pH). The name "collagenase" was initially introduced to describe an enzyme activity that was produced from cultured tissue explants of developing tadpoles and that broke down collagen fibrils that were previously highly proteinase-resistant. This discovery was ground-breaking. It was the first instance of an activity that could totally dissolve and cleave natural collagen fibrils while working at neutral pH. It signaled the start of a fascinating period of research that resulted in the finding of numerous unique matrix metalloproteinases (MMPs), which range from six to seven in number and have the ability to break down collagen in a variety of in *vitro* circumstances (GROSS & LAPIERE, 1962).

Since collagenase does not damage cell membranes, it has long been widely employed in laboratories for cell dispersion, tissue separation, and cell culture. The collagenase enzyme is divided into four categories. The most prevalent collagenases may be extracted from both microbial and animal tissues, the sample is isolated and characterized (Alipour *et al.,* 2016).

3. Collagenase structure

3.1. Secondary structure

Collagenase has a secondary structure that predicts how many helices, sheets and amino acids are in it see (Figure 2, a). This secondary structure has a big effect on how the protein is folded. Using PSIblast-based secondary structure prediction (PSIPRED) and the predict protein service, the existence of helix, sheet, and turn in different collagenases was predicted. Users can conduct genuinely scaled biological investigations using the PSIPRED web server's numerous protein annotation tools. Sequences of collagenase protein are examined with DISOPRED and TMHMM tools, which respectively, look at transmembrane proteins and disordered proteins (Szklarczyk *et al.,* 2015).

3.2. Three-dimensional structure

Collagenase is a protein that can be used to predict three-dimensional protein models. The collagenase amino acid sequence from *Bacillus cereus* MH19 was chosen. The Swiss-Model tool predicts the target protein's three-dimensional structure as shown in (Figure 2, b) (Hisano *et al.,* 1989) . Using the most relevant template, we were able to predict a protein model of the same type of collagenase made by the bacterium *Bacillus cereus* MH19.Structure analysis and verification server (SAVES) assessed and verified the predicted collagenase protein model.

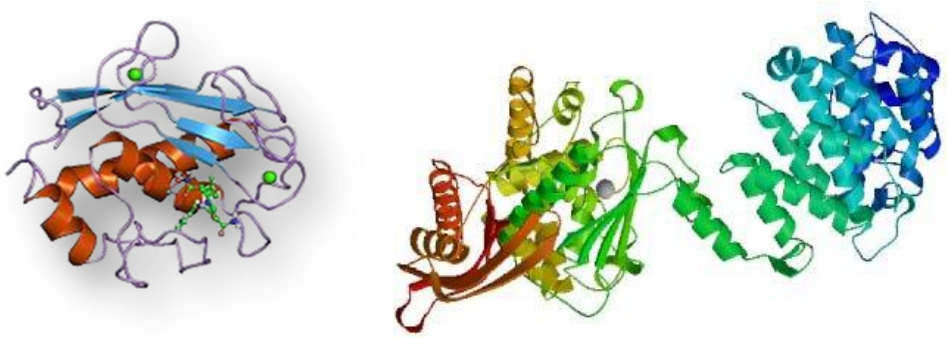

Fig. 2: A) Collagenase structure B) SWISS three-dimensional models of BCC000504 collagenase.

4. Characters of collagenase

The unit activity of collagenase after collagen has been hydrolyzed is represented as mols of the amount of L-leucine and glycine equivalents that are released per minute per millilitre of enzyme since collagenase enzymes are specific for the collagen substrate (MOORE & STEIN, 1954) .

A specialized protease known as collagenase is capable of hydrolyzing either denatured or native triple helical collagen. In light of its potential industrial and biological applications. Bacterial collagenases can differ substantially based on variances in their structural and functional characteristics. The precise structural properties of collagenase make it difficult to understand how it breaks down collagen (Rani & Pooja, 2018) . These enzymes are characterized by their thermostable, alkali resistance (Johnvesly & Naik, 2001; L. Zhang *et al.,* 2019) .Also collagenases have the ability to break down fibrillate-like types Ⅰ, Ⅱ, and Ⅲ into smaller pieces. Some other types of collagenases, like MMPs (MMP-2 and MMP-14), also break them down. Collagenases can also break down other proteins that make up the extracellular matrix (ECM), as well as other proteins that don't (Nagase & Murphy, 2013).

Collagenase is characterized via electrophoresis, mostly through estimation of molecular weights. According to the kind of enzyme (metallocollagenase or serine), microbial or animal tissue source, and these variations are reflected in the reported molecular weights (Daboor *et al.,* 2010).

The molecular weight of two collagenases from the bacterium *Clostridium histolyticum* molecular weight 105 and molecular weight 57 kDa respectively were discovered (Harper *et al.,* 1965;Bond & Van Wart, 1984).Also from the same species, six distinct collagenases with molecular weights varying from 68 to 128 kDa were identified demonstrated that the molecular weights of collagenases isolated from a similar strain of *Clostridium perfringens* ranged from 80 to 120 kDa. It appears that collagenases produced by bacteria normally have molecular weights greater than 55 kDa, but collagenases made from animal tissues typically have smaller molecular weights (Matsushita *et al.,* 1994).

5. Collagenase classification and types

There are four classes of collagenase (I, II, III, and IV) and additional substrates besides collagen (Table 1) (Alipour *et al.,* 2016) . Based on their various physiological roles, they are often classified as either serine collagenase or metallocollagenase types of collagenases.

5.1. Serine collagenases

Serine collagenases contain a serine residue at their catalytic sites, just like all other serine proteinases. They usually have a molecular weight between 24,000 and 36,000 kDa (Roy *et al.,* 1996) . They are frequently engaged in hormone production, protein degradation, blood-clotting, and fibrinolysis, and they have the ability to the splitting of the triple helical structure of collagen Types I, II and III (Neurath, 1981).

5.2. Metallocollagenase

Matrix Metalloproteinases (MMPs) are metallocollagenases with molecular weights between 30,000 and 150,000 KDa (Krashen, 1982). Metallocollagenases are frequently found in fish and

animal tissues, including the hepatopancreas and bones, fins, and skins of marine crabs (Sivakumar *et al.,* 1999).

Table 1: Classification of collagenase and its substrate (Alipour *et al.,* 2016) :

Enzyme	Matrix metalloproteinase	Substrate
Collagenase Ⅰ	MMP-1	Collagens 1, 3, 7, 8, 10, gelatin, L-selectin, interleukin-1, entactin, ovostatin, MMP-2, MMP-9, proteoglycans, aggrecan
Collagenase Ⅱ	MMP-8	Collagens 1, 3, 5, 7, 8, 10, fibronectin, gelatin, aggrecan
CollagenaseⅢ	MMP-13	Collagens 1, 4, 9, 10, 14, fibronectin, MMP-9, gelatin, plasminogen, aggrecan, perlecan osteonectin
Collagenase Ⅳ	MMP-18	Type I collagen

6. Collagenase source

6.1. Animal collagenase

Animal collagenase breaks down the triple helix of collagen at a particular location (Pal & Suresh, 2016) . It is widely reported to extract, purify, and characterize collagenase of animal origin. The majority of reports come from fish viscera (Murado *et al.,* 2009; Sovik & Rustad, 2006; Villamil *et al.,* 2017) .Collagenases from animal systems are restricted because of their complex systems, which raises the cost of purification due to their lack of site-specific action (Jhample *et al.,* 2015).

6.2. Plant collagenase

According to past findings, the majority of collagenases are said to have both microbial and mammalian origins. There are not many reports about plant collagenases (Kim *et al.,* 2007; Raskovic *et al.,* 2014) . Plant collagenases work on native collagen in a manner similar to that of animal collagenases. Plants' capacity to make collagenase plays a big part in defense against pests (T.R. Gomes *et al.,* 2011). Collagenase from fig and ginger has been isolated and characterized (Kim *et al.,* 2007; Raskovic *et al.,* 2014). Because plant systems are just as complex as animal systems, large-scale plant collagenase production is even more limited (Jhample *et al.,* 2015).

6.3. Microbial collagenase

The broad substrate specificity of microbial collagenases allows them to break down both water-soluble and water-insoluble collagens in their triple helical regions at X-Gly links.

6.3.1. Bacterial collagenase

The first collagenase to be produced by a microbe was from the pathogenic *Clostridium* sp. (Pal & Suresh, 2016). Similar to this, certain other pathogenic bacteria have been reported to produce collagenase (Houle *et al.,* 2003; Jung *et al.,* 1999; Miyoshi *et al.,* 2008). Currently, the majority of collagenase produced at an industrial level comes from *Clostridium* sp. Also, Collagenase derived from *Vibrio alginolyticus* is a new enzymatic technique for separating stem cells and producing high-quality results quickly. Cellular yield, proliferation, and clonogenic capacities were first compared in order to study the enzyme concentration and incubation duration. The phenotypic characteristics of the optimized protocol were studied, and its mesodermal lineage differentiation potential was assessed. Then, such procedure was contrasted with two collagenases based on *Clostridium histolyticum* (Quintero Sierra *et al.,* 2023).

6.3.2. Actinomycete collagenase

Thermoactinomyces sp. is a thermophilic actinomycete strain, was discovered to produce a highly thermostable serine collagenase (Petrova *et al.,* 2006). Maximum collagenase activity was obtained by *Streptomyces exfoliatus* CFS 1068, a cultivable field soil isolate (Jain & Jain, 2010).Microbial collagenase enzyme expressed in *Streptomyces violaceoruber* underwent a safety assessment (Harazono *et al.,* 2020).

6.3.3. Fungal collagenase

Collagenase is one of the most important enzymes used in the processing of raw meat. The *Aspergillus awamori* 16 and *Aspergillus awamori* 22 fungi were grown together for 30 days in

submerged settings utilizing a novel method of cultivation including the immobilization of fungal cells (Blieva *et al.,* 2020). *Penicillium* sp., isolated from a sample of destroyed leather, was investigated for collagenase production (Kate & Pethe, 2022).

Currently the application of this microorganism's collagenase is constrained by its pathogenic and anaerobic characteristics as well as its capacity to produce toxins. Researchers are eager to identify alternative microbial sources that are either non-invasive or less harmful and capable of producing this enzyme in an economical manner is driven by the possibility of an outbreak of such a microorganism and the rising cost of enzyme production as a result (Bhagwat *et al.,* 2015). As a result, numerous scientists are now pursuing the screening and isolation of high enzyme-yielding microorganisms by using newer and original applications, we can make collagenolytic enzymes more cost-effective by optimizing medium components (Bhagwat *et al.,* 2015; Bhagwat & Dandge, 2016; Ferreira *et al.,* 2017). Microbial production is always better than animal or plant production because they have fewer needs and are more productive (Jhample *et al.,* 2015).

7. The most physico-chemical factors that effect on collagenase production

There are some factors that are needed by microbe to produce enzymes. It can be physical factors such as temperature or chemical factors such pH and all frequently adjusted factors are summarized in (Figure 3).

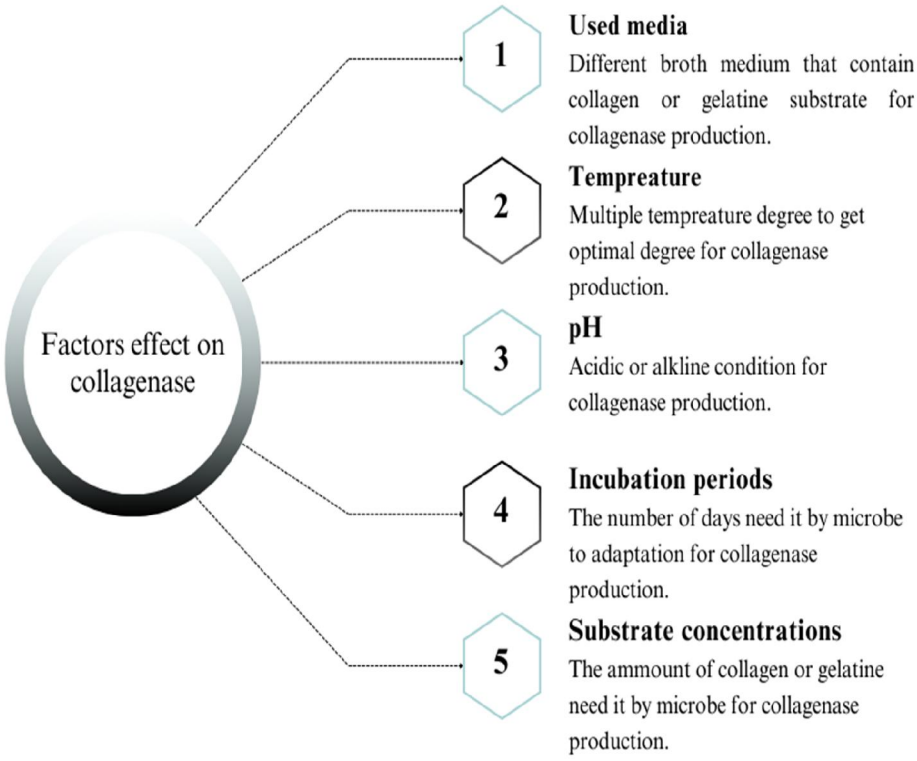

Fig. 3: The most common factors that affect collagenase production in recent research.

8. Mechanism of collagenase

In the development of the embryo, the morphogenesis of the organs, and the remodeling and repair of tissue, the breakdown of triple-helical interstitial collagens is crucial. The prototypical collagenase, MMP-1, has a three-dimensional structure that shows that the enzyme's substrate-binding site (SBS) is not large enough to support three-dimensional collagen see (Figure 4, a and b). Before hydrolyzing the peptide bonds, collagenases help to hold the triple helical structure in place by binding and unwinding it locally. Before it breaks the collagen I bond, it first joins and then breaks the hard triple helical substrate (Chung *et al.,* 2004).

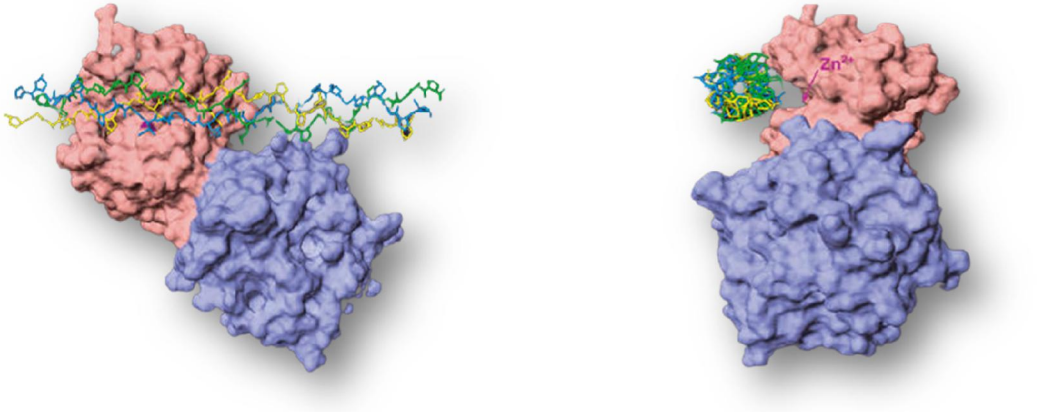

Fig. 4: Three collagen triple-helical peptides are presented in figures **a**nd **b**. In figure **a**, the active site of porcine MMP-1's catalytic domain is manually aligned with the triple-helical peptides, and in figure **b**, the active site has been rotated 90 degrees to the left. Purple: zinc ion, blue: HPX, and pink: catalytic domain modified from (Kramer *et al.,* 2001).

9. Application of Collagenase

Microbial collagenases have been thoroughly researched from a variety of sources and have practical uses all around the world and it has been summarized in (Figure 5). Due to their extensive use in a variety of industries, including the pharmaceutical, food, livestock, tannery, cosmetic, and bio-restoration of frescoes, microbial collagenases are of utmost importance (Pal & Suresh, 2016; Ranalli *et al.,* 2005; Watanabe, 2004).

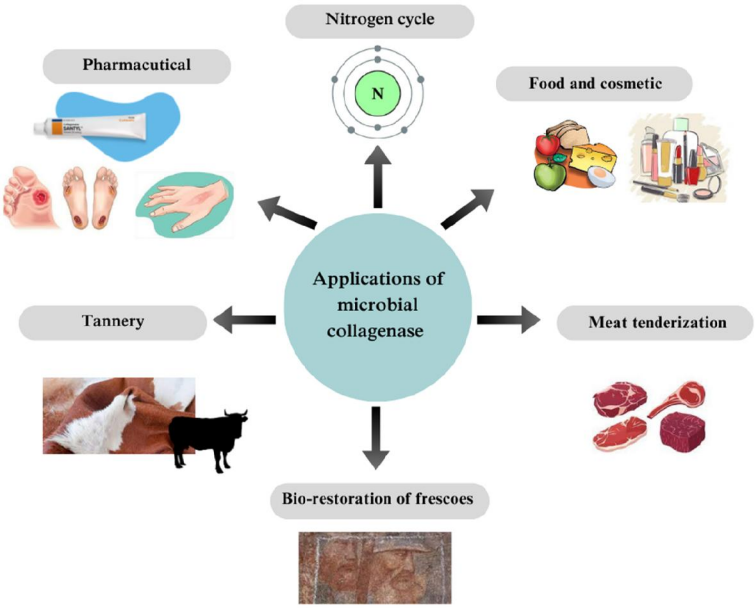

Fig. 5: Worldwide application of collagenase.

9.1. Medical field

There are numerous uses for collagenases in the medical field. Collagenase plays a critical role in the migration of cells and the remodeling of collagen during the process of wound healing and tissue regeneration (Agren *et al.* 1992).

9.1.1. Dupuytren's disease

Dupuytren's disease results in an unnatural thickening and loss of function of the tissue as a result of an excessive buildup of collagen tissue. The condition, which frequently affects elderly white men, is probably inherited (Witthaut *et al.,* 2013; Peimer *et al.,* 2015). The FDA has been approved the use of collagenase from *C. histolyticum* and proposed as a novel approach to treat Dupuytren's muscle contractions (Gur *et al.,* 2011).

9.1.2. Wound healing

Experimental studies have demonstrated that collagenase enzymes can speed up dermal cell proliferation, angiogenesis, and migration during the healing of wounds (McCallon *et al.,* 2014). collagenases kill the living cells in necrotic tissues around wounds while leaving healthy tissue unharmed.

9.1.3. Burns

Burns are a significant global public health issue. The most common causes of death are burns from hot liquids, steam, electrical burns, and other burns. for which there are no reliable statistics globally. In low- and middle-income areas, burns caused by fire account for over 96% of fatalities. Recent research has shown Collagenase ointment has been shown to reduce the time it takes for burn wounds to heal. A study compared 78 patients with burn wounds who were treated with collagenase *C. histolyticum* and 41 patients with burn site tissues that had been removed surgically (McCallon *et al.,* 2014).

9.1.4. Debridement

To improve healing, harmful tissue is removed from a wound through debridement. This treatment, which is essential for preparing the wound bed, can be carried out by removing the tissue surgically, chemically, mechanically, or autolytically. Microbial collagenases *C. histolyticum* in the health industry one of the most common uses for microbial collagenases is in wound and other injury enzyme debridement when devitalized tissues need to be removed. Debridement is the process of removing foreign objects and diseased or contaminated tissue from the area surrounding a lesion. This method is used to treat wounds and burns, leg ulcers, chronic, slow-healing wounds, and wounds that are thought to be stuck in the inflammatory phase of wound healing (Ramundo & Gray, 2008).

9.2. Animal Tissue Culture (ATC):

A crucial tool with widespread use in the fields of molecular biology, biotechnology, and the pharmaceutical industries is cell culture. The use of microbial collagenase in ATC procedures is very common. Extracellular matrix, which surrounds cells and contains a high concentration of collagen proteins, must be disaggregated in order to utilize the cells. Trypsin, is frequently used to separate animal tissues, has a restricted pH range of 7.2–7.4 and is extremely temperature-sensitive (Manjusha *et al.,* 2013). Microbial collagenase, on the other hand, is active in a broad range of pHs and temperatures, making it a useful enzyme source in ATC domains. Many studies have shown that microbial collagenase acts as a cell dislocator in cell culture (Bhagwat & Dandge, 2016;Manjusha *et al.,* 2013).

9.3. Meat tenderization

There are many different ways to eat meat. The roughness of the meat, which is mostly to blame for the unacceptability of meat products, is caused by a higher concentration of skeletal muscles (Kemp *et al.,* 2010) . Mechanical tenderization is a technique used to make meat more tender, but it can often cause meat products to become contaminated with *Salmonella typhimurium* and *Escherichia coli* (Echeverry *et al.,* 2009) The use of protease from both microbial and plant sources is another way for meat tenderization that has been identified. The broad specificities of the proteases

used in this procedure toward meat proteins may lead to the formation of undesirable sensory qualities in processed meat products (Pal & Suresh, 2016) . The utilization of microbial collagenases, which are capable of specifically breaking down collagen, is an effective technique for rendering meat products more tender. The ability of collagenase from *Clostridium histolyticum* and *Vibrio B*-30 to tenderize meat has been documented (CRONLUND & WOYCHIK, 1987). However, the utilization of these species' collagenases is constrained by their pathogenicity. *pseudoalteromonas* sp. SM9913 collagenase and *pseudomonas* sp. SUK collagenase, which is non-pathogenic and is active at extremely low temperatures, has been used in several publications. Meat can be tenderized with these kinds of enzymes while still being refrigerated, reducing the risk of microbial infection (Zhao *et al.,* 2012;Bhagwat & Dandge, 2016).

9.4. Leather industry Microbial

Collagenase produced by microorganisms may be used in the leather industry. After tanning, collagenase will open up the fibrous network in the skin, allowing the color to spread more freely. This process improves the leather's softness, texture, and overall appearance, as well as improving the absorption of dye into the skin (Kanth *et al.,* 2008).

9.5. Bio-restoration of frescoes

A fresco is a type of ancient painting. These monuments' surfaces were vulnerable to black crusts, sulphation, nitration, hydrocarbon deposition, and dust due to rising pollution. Additionally, organic matter that is placed during restoration but left behind thereafter likewise modifies stonework. The significant number of organic compounds, including glue and casein, serve as an excellent substrate for the growth of microbes and mycetes, which ultimately deteriorate the painting's surface. Compared to other examined proteases, the highest removal rate for organic residues was achieved by the utilization of collagenase derived from the bacterium *Clostridium histolyticum* (Ranalli *et al.,* 2005). Microbial collagenase may therefore be employed to help in the bio-restoration of frescoes.

9.6. Extraction of collagen

Multiple sources have reported collagen extraction [61]. Acidic solution is typically utilized in this extraction method. Following acid extraction, the residual biomass still included a significant amount of collagen (Bhagwat & Dandge, 2016). This collagen can be economically recovered using microbial collagenase. Collagen from salmon skin has previously been recovered using collagenases from *Bacillus cereus* and *Klebsiella pneumonie*. Collagen recovery was higher when collagenase and acid extraction were combined than when acid extraction was used alone (Suphatharaprateep *et al.,* 2011).

9.7. Preparation of collagen peptides

Usually, collagen undergoes proteolysis to produce collagen peptides. Numerous proteases have been documented to hydrolyze collagen, producing collagen peptides that range in size from 0.5 to 20 kDa (Pal & Suresh, 2016). The United States Food and Drug Administration (FDA) has approved the consumption of collagen peptides that have been formed (Bilek & Bayram, 2015). Collagen hydrolysates are naturally active and have antioxidants, antibacterial, anti-fatigue, anti-cancer, immunomodulatory, neuroactive, mineral, and hormone-regulating capabilities as well as ACE inhibitory traits. Due to their wide range of qualities, including their capacity to hold water, their ability to bind fat, their ability to stabilize foam, their swelling, solubility, and their emulsifying capabilities, it can also be used by the food, pharmaceutical, and cosmetic sectors (Halim *et al.,* 2016;Pal & Suresh, 2016). The prevention of arthritis, osteoporosis, stomach ulcers, and hypertension is another benefit of collagen peptides (Ku *et al.,* 1993) . As a result, collagen peptides play a crucial role in the food and beverage sectors. In previous publications, collagen hydrolysates produced by the enzyme collagenase from *Penicillium aurantiogriseum* URM4622 had antibacterial and radical-scavenging activities (Lima *et al.,* 2015) . As opposed to squid skin collagen hydrolysates, which exhibited antioxidant, anti-hyaluronidase, and anti-tyrosinase activity (Nakchum & Kim, 2016).

9.8. Global nitrogen cycle Worldwide

Massive amounts of marine garbage are created globally, and both the population and fish production are gradually rising. Similar to chitinous waste, however, collagenous waste is destroyed by naturally occurring microbes with collagenolytic capacity, therefore it does not collect as much in ocean sediments or the surrounding area (Zhang *et al.,* 2015 ; Pal & Suresh, 2016). The nitrogen cycle on a global scale is essential for sustaining life on earth and is partially completed by the microbial breakdown of collagenous waste (Zhang *et al.,* 2015).

10. Future perspective

There is some hope that further research will lead to the development of techniques and procedures for collagenase production from novel sources. Few microbial species have so far been able to purify and describe collagenases, which restricts the use of collagenase from *Clostridium* sp. is a species that produces anaerobic pathogenic toxins. Therefore, there is a chance that this microorganism will spread, and the cost of producing enzymes will rise as a result of researchers' desire to find alternative sources of aerobic non-pathogenic or less pathogenic microorganisms. It is also possible that there are many other microbial collagenases that are not yet identified or purified. Additionally, future research should focus on using fish waste-derived collagen in the food and pharmaceutical industries. Additionally, it would be fascinating to investigate how collagenase can be used to prepare bioactive collagen peptides and hydrolysates for use in the pharmaceutical and food sectors.

11. Conclusion

Due to their great specificity at extremely low concentrations and highly selective nature, collagenases have significant potential in therapeutic activities. Under various physiological pH and temperature circumstances, collagenases cleave the helical portions of collagen molecules into fibrillar form. There are numerous industrial uses for collagenase. Recent research indicates that they are increasingly utilized in the food industry, pharmaceuticals, and other sectors. The ease of availability of microbial collagenases makes them a current topic of research. Due to data shortages and the need for additional research, collagenase enzyme looks to be made and utilized as a drug in clinics in the near future for the treatment of burns, wounds, and some other ailments, according to its recently proposed application.

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