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Microbial collagenases: Challenges and prospect in production and potential applications in food and nutrition

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Abstract

Microbial collagenase is a promising enzyme in view of their extensive industrial and biological applications. The emerging evidence of potential food application and health benefits revealed that microbial collagenase shows significant promise as a main component for bioactive functional ingredients/peptides preparation. Collagenases are the important virulence factors, which play a crucial role in the global degradation of the extracellular matrices of animals, due to their collagen degradation ability. There is a lack of scientific consent for a well-defined and proper screening of collagenase-producing microorganisms. A vast controversy was found in the literature regarding the correct identification of microbial collagenase. This review summarizes the current technologies and strategy used to improve the screening, production, and purification of microbial collagenase with a comprehensive insight especially focusing on the classification, structures and collagen-degrading mechanisms of M9 family representative collagenase. It also highlights the potential of microbial collagenase to develop a process for meat tenderization and bioactive "true" collagen peptides or hydrolysates preparation. In addition, the critical challenges and various strategies for potential applications of collagenase in food, nutrition, biotechnological and medical sectors are highlighted.

Keywords

Collagen; Microbial collagenase; Collagenase production; True collagen peptide; Food applications

1. Introduction

Collagens are the major structural proteins of connective tissues such as skin, bone, cartilage, tendon, and blood vessels of mammals. Collagen is composed of three α-chains of predominantly repeating Gly-X-Y triplets sequences (X and Y are often proline and hydroxyproline), which induce each α chains to hold a left-handed helix with remarkable

primary, secondary, tertiary and quaternary structure[1-4]. There are 29 distinct collagens, and each collagen differs considerably in their amino acid sequence, structure, and function[1-3, 5-6]. The proteolysis of collagen is essential for numerous physiological functions, including tissue remodeling, morphogenesis, and wound healing. Collagen has been recognized as an essential contributing factor in numerous pathologies, such as tumor cell spreading, arthritis, tissue ulcerations, cardiovascular disease, neurodegenerative diseases, and periodontal disease[1-3, 6]. The commercial applications of collagen and collagen derived products are increasing at a rapid rate as an ingredient in beverages, foods, drugs, cosmetics, and a variety of health care products[4, 7-10]. Due to the decrease in body collagen synthesis, its demand for the skin, hair and bone tissues increases with aging. It has been reported that collagen peptides, a product of collagen degradation possess several biological activities of industrial, food, nutritional and biomedical interest[8, 29-30]. Furthermore, collagen degradation products reportedly have various activities of medical and pharmaceutical interest: agents for osteoporosis, gastric ulceration, hypertension, skin moisturizers, and preservatives. Collagen degradation products can be added to food and beverage products to improve their functional and nutrition properties without causing any production problem due to its low viscosity and high solubility in water[7].

Type I collagen is the most abundant and commercially important type of collagen and used widely in food, biomedical, pharmaceutical and cosmetic industries due to its excellent cell adhesion properties, high biocompatibility, biodegradability, and weak antigenicity[4, 7-12]. Collagen and their fibril with another polymer can also be used to prepare the porous scaffolds. These scaffolds have been extensively used in tissue engineering and biomedical sectors[13-19]. At present, the skins and bones of land-based animals (bovine and porcine) are the chief sources of commercial collagen. Though, epidemics of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and

foot-and-mouth disease (FMD) have brought an anxiety among consumers of collagen and collagen-derived products from these land-based animals[4]. Further, collagens and collagen-derived products from bovine and porcine are forbidden in some religions[4]. Consequently, the demand for realistic collagens from substitute sources, particularly from aquatic/marine origin has been increasing over the year[4].

Enzymes have been used an integral part of the various industries for a long time. Enzymatic methods constitute an essential and important part of the production process due to highly specific nature, high activity at very low concentrations under mild conditions of pH and temperature, which may also result in fewer unwanted side-effects and by-products[20]. Microbial collagenases are secreted by anaerobic/aerobic pathogenic and non-pathogenic microorganisms to utilize collagen as a source of nutrients[21]. The 'native' collagens are resistant to most common proteases due to their triple-helical structure but are readily cleaved at a specific site by collagenase. Many enzymes that have been originally described as collagenases were shown later to be either proteases or peptidases of broad or different specificity. The collagenases are found in animals (EC 3.4.24.7) and microorganisms (EC 3.4.24.3), and differ each other in their substrate specificity. Animal collagenase splits/digest the native triple-helical collagen at a single peptide bond[37]. However, microbial collagenase is unique and capable to hydrolyze native collagen under physiological conditions. They possess broad substrate specificities to degrade both water-insoluble 'native' collagens and water-soluble denatured ones[27]. Due to this unique activity, microbial collagenases play important roles in embryo development, morphogenesis, tissue remodeling, wound healing, and human diseases, such as arthritis, cancer, and atherosclerosis[6, 28]. Therefore, microbial 'true' collagenases have gained much attention on their numerous industrial, biotechnological, pharmacological, medicinal and food applications[3, 8-10, 21].

Collagenases are used in various sectors such as food, tannery, fur, fish processing, brewing, meat processing, clarification and stabilization of beer, medicine, cosmetics, fish silage, fish sauce, fish meal, in the processing of animal feedstuffs as well as in scientific and analytical research[21, 31-34]. Hence, screening of high yielding enzyme-productive microorganisms and development of low-cost cultivation media, as well as different cultivation methods for economic production of the collagenolytic enzymes with novel properties are being intensively pursued by scientific community[9, 21, 36]. In fact, this review is intended to highlight pertinent information related to overview about microbial collagenases and the trends and prospects for future, with special emphasis on the outlook for their potential applications in food industries. This review also discussed the novel strategies that can be used for novel functional foods and beverage products development using collagen hydrolysate.

2. Collagenases: Occurrence and distribution of collagenase

Collagenases have been usually recognized as enzymes that specifically attack 'native' collagens and water-soluble denatured ones[21, 27]. The term 'true collagenase' was coined on the basis of their capacity to hydrolyze/digest native bovine achilles tendon collagen[21]. The collagenases are mainly found in animals, microorganisms and plants (Fig 1). The occurrence of collagenases in plants also has been reported[22-23, 36].

Collagenase is one of the most widely used enzyme in several applications. In the past, much attention has been given to isolation and extraction of collagenases from animal tissues. Recently, the emphasis has shifted to obtaining microbial collagenases, which are advantageous in comparison with animal's collagenase[8-10, 21, 45-46]. At the industrial scale, this enzyme is obtained from pathogenic microorganism *Clostridium* sp. Weinberg and Randin reported the first evidence for bacterial collagenase production in 1932[21]. In 1937,

Maschmann suggested the name collagenase for an enzyme from *C. perfringens* that can digest both 'gelatin' and 'collagen'[21].

2.1. Plant collagenase

Collagenases are mostly found in animal and microorganisms. Collagenase is also reported from few plants[22-23, 36]. The plant collagenases preferentially cleave the native form of collagen at a specific site. These enzymes have not been shown to be involved in biological processes, but may be related to a biological function such as defense against pests such as nematodes[22]. The collagenolytic activity of plants may play a significant role as a line of defense against environmental changes, and their activation can be triggered by applying various types of stress[22]. The isolation and characterization of collagenase from the ginger rhizome *(Zingiber officinale)*[23], fig (*Ficus carica* var. Brown Turkey) latex[36] have been reported.

2.2. Animal collagenase

Animal collagenase (EC 3.4.24.7, Interstitial collagenase) splits/digest the collagen at a single peptide bond across the three α chains organized in a native triple-helical structure. Animal collagenase can cleaves the triple helix of collagen at about three-quarters of the length of the molecule from the X-Gly bond. The collagenase is widely distributed in vertebrate animals. In animal collagenases, the degradation of 'native triple helical collagen' or 'water-insoluble native collagen' significantly depends on the species of origin and collagen types [21, 31-32, 49]. Collagenases from the animal origin were frequently extracted, and purified from fish processing byproducts or viscera of fish[47-48] and other animals[21, 32, 44]. A review on collagenases from fish processing byproducts has been reported[32].

2.3. Microbial collagenase

Microbial collagenases (EC 3.4.24.3) digest the native collagen in their triple helical region at X-Gly bonds[10, 31-32]. Microbial collagenases possess broad substrate specificities and

degrade both 'water-soluble/insoluble native' and 'denatured collagens' in its triple helical conformation[27, 50]. Microbial collagenases can degrade each polypeptide chain of collagen at multiple sites. Production of microbial collagenase has been reported from some pathogenic bacteria and fungi (Fig 1). Bacterial sources have been widely used for the production of microbial collagenase[9-10, 27, 34, 46, 51-52]. First identified and characterized microbial collagenases was produced by *Clostridium* sp. The main source of our knowledge on microbial collagenases came from the extensive studies of *C. histolyticum* collagenase[2, 28, 53-56]. Seven different polypeptides with collagenolytic activity have been demonstrated in *C. histolyticum* and divided into two groups on the basis of substrate specificity[55].

Among the well-studied collagenase-producing microorganisms *C. histolyticum*, some other clostridia can also produce collagenase, but in literature, little information was found regarding their biochemical properties[55]. *C. perfringens* and *C. tetani* are the collagenase-producing other clostridia. They are extensively distributed in nature, particularly in soil and water contaminated with feces. They also live in the intestinal tracts of humans and animals[55]. Sometimes, *C. perfringens* causes infection, representing the most common pathogen of *Clostridial* histotoxic infection[55]. It was reported that pathogenic strains such as *C. histolyticum* and *C. tetani* use collagenase to facilitate the host invasion, colonization, and toxin diffusion during anaerobic infections[2]. Instead, one of the other well-investigated bacterial collagenases is *Vibrio alginolyticus* collagenase. The collagenase activity of *V. alginolyticus* collagenase was found higher than that of any other bacterial collagenase[71-72, 75]. In recent year, much emphasis has been shifted to microbial collagenases as compared to animal's collagenase[8-10, 21, 45-46]. To date, microbial collagenases have been purified from few species, and their genes have been cloned and sequenced[57]. However, many microbial collagenases have not yet been enzymatically and structurally characterized[57].

3. Commercial availability of microbial collagenase

The first commercially available microbial collagenase was isolated from *C. histolyticum* and for a long period, it was only commercially available microbial collagenase. These *Clostridial* collagenases were well studied and characterized with their properties. Still, they are the only reference enzymes for the comparison of newly discovered collagenolytic enzymes[21]. These enzymes are the important virulence factors in a variety of pathogenic microorganisms. *Clostridial* collagenases are large multi-modular zinc-metalloproteinase of approximately 115 kDa, consisting of four to six domains. Two to four accessory domains of approximately 10 kDa each forms a C-terminal collagen recruitment unit of variable composition, providing important exosites for native collagenolysis, responsible for collagen binding and swelling[2]. Although, most *Clostridial* strains possess one collagenase, *C. histolyticum* encodes for two collagenases with complementary characteristics: collagenase G (Col G) exhibits high collagenolytic and low peptidolytic activity. However, its homologue collagenase H (Col H) shows low collagenolytic and high peptidolytic activity[2].

4. Isolation and screening of collagenase-producing microorganisms

Collagenase producing microorganisms have been found in a diverse habitat such as soil[33], thermal regions[39-41], gut debris of earthworms[34], food materials, fish sauces[43], fish byproducts/waste (skin, bone, fins, etc.) material[32], soil and sewage samples of the slaughterhouse, various pathological sources and leather house/industry[21, 44].

Several methods are reported by various research groups to screen the collagenase-producing microorganism. However, there is a vast scientific controversy was found in the literature regarding the appropriate and well-defined screening of the collagenase-producing microorganism. This controversy was found due to similar enzymes such as collagenolytic protease, gelatinase and other protease[29, 34, 45-46, 51-52, 54, 58]. There is a lack of a well-defined standard method for the isolation and screening of collagenase-producing microorganisms on

the basis of qualitative and quantitative properties. Thus, more research should be focused on the standardization of the rapid, authentic and cost effective qualitative screening of the collagenase-producing microorganisms. Here, we discussed a number of methods reported by the different research team for the screening of collagenase-producing microorganisms.

Based on gelatin hydrolysis approach: Gelatin is a class of protein fractions that have no existence in nature but are derived from the parent protein collagen by denaturation. The gelatin hydrolysis can confirm the qualitative determination of collagenase activity. Several researchers have reported the use of 1-3% gelatin as a supplement in different medium compositions such as trypticase soy agar, selective medium, potato dextrose agar, and nutrient agar to screen extracellular collagenase-producing microorganism[9-10, 45-46]. Collagenase production was evident as a clear or clear halo zone around the colonies[29, 34, 44-46, 51-52, 58]. However, the clarity of hydrolyzed zone may not be very sharp on the media plate. Therefore, the clarity of the hydrolyzed zone around the microbial colony can be improved by precipitating the proteins with trichloroacetic acid (TCA)[59]. It was reported that application of 30% TCA produced a very sharp and clear zone gelatin hydrolysis[59]. An agar cup method was also reported to screen the collagenase-producing microorganisms[46]. Cell-free supernatant were placed into cup-plates (8 mm) containing 1-2% (w/v) gelatin. The ability to digest gelatin was demonstrated by the formation of transparent halos on cup-plates and expressed in mm[46].

Based on hydrolysis of collagen and collagen hydrolysate approach: The collagenolytic activity of microorganisms could be detected using insoluble/soluble collagen or collagen hydrolysate products[60]. Mostly collagens have low denaturation temperature (30-37 ^{o}C). To avoid the collagen denaturation, collagen supplemented media cannot be sterilized by autoclaving. Therefore, the sterilization of growth medium supplemented with collagen is a challenging task for the scientific community[60]. An aseptic addition of collagen into the

sterilized growth medium may be problematic due to the non-sterility of the collagen. Furthermore, the acidic collagen solution (collagen usually dissolve in dilute acid, 0.5 M acetic acid) may alter the pH of the medium. To overcome these problems, various other sterilization techniques have been used to disinfect and/or sterilize the resuspended collagen such as propylene oxide, ethylene oxide, gas plasma sterilization, gamma radiation, chloroform, glutaraldehyde, formaldehyde, acidic pH, electron beam sterilization, or peracetic acid sterilization, or combinations[61-63]. These sterilization techniques do not adversely affect the structure and bio-tropic properties of the source material/collagen[62-63].

5. Measurement of collagenase activity

Microbial collagenase can act on both water-insoluble 'native' collagens and water-soluble denatured collagen (i.e., gelatin). Collagenase activity is influenced by various factors such as solubility, viscosity, the concentration of collagen solution, type of acid used for dissolving the collagen, the amount of enzyme, collagen degradation products such as hydroxyproline, amino acids, peptides, and other reaction conditions (pH, temperature, and agitation). Water insoluble and soluble collagen are mostly used as substrates for the collagenase assays[40-42]. In these methods, quantitative estimation of amino acid produced from the collagen substrates is commonly used to determine collagenase activity[64, 138]. Rosen's modified colorimetric ninhydrin method for the quantitative estimation of amino acid was usually used to measure the collagenase activity[138]. In this method, a collagen substrate (usually 'native' collagen) incubated with enzyme and collagenase activity was determined by measuring the free amino groups released from the collagen substrate. The collagenase activity is usually expressed as μmoles of amino acids released per min/mL under standard assay conditions using L-leucine/glycine as the reference standard. However, some research groups also reported the determination of collagenase activity using the gelatin as an assay substrate[52, 60].

A modified ninhydrin-based method incorporating polyethylene glycol (PEG) for the assay of collagenase activity by spectrophotometry was demonstrated[64]. PEG is series of polyether compounds that can stabilize Ruhemann's purple efficiently during ninhydrin involved reaction, and the coexistence of PEG makes a ninhydrin-based reaction suitable for collagenase assay. The absorbance was increased with the increased in digestion time. Most of the collagenase assays are time-consuming (3-18 h)[64]. If the collagenolytic assays conducted at more than 37 ^{o}C, (a temperature above to the denaturation temperature of collagen), that is most probably measurement of the gelatinolytic activity instead of the collagenolytic activity[21]. At high temperature, collagen is hydrolyzed, and collagen hydrolysate or gelatin had certainly occurred. Therefore, various synthetic peptides such as 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide), N-[3-(2-Furylacryloyl)]-L-leucyl-glycylL-prolyl-L-alanine (FALGPA), and azo dye-impregnated collagen were developed as substrates for efficient quantification of collagenase activity[8-9, 41-42, 45, 65].

In azocoll assay, $CaCl_2$ is used as a cofactor and a reaction time of 3-5 h at a maximum temperature about 37 °C. In this assay, one collagenase activity unit (U) is defined as the amount of enzyme/mL that produced an increase in the optical density of 0.1 at 520 nm, due to the formation of azo dye linked soluble peptides[9-10]. Radiolabeling with 14C, 2H, or 3H or fluorescence-labeled collagens was also developed as a substrate for quantitative estimation of the collagenase activity[64]. These methods are considered more convenient and sensitive assay for collagenase activity. However, these methods require expensive devices and lead to the generation of radioactive waste. Fluorescent collagens and synthetic peptides as substrates seem more attractive, but labeling with fluorescein isothiocyanate (FITC) or biotin is a time-consuming and expensive process[64].

A sensitive, fast and specific electrophoresis method for the determination of collagenase activity is also described[67]. In this zymography method, collagen is used as the substrate. Collagen is incorporated in the polyacrylamide gel and carried out the non-denaturing electrophoresis. This method represents the hydrolysis of collagen by the enzyme and to be detected as clear zones/bands surrounded by the dark background of the stained protein (collagen). This method can detect the collagenase activity up to nanogram levels. The collagenase activity band can be visualized by staining gel with coomassie blue dye. Unexpectedly, there is a number of collagenase assay - still a simple, reliable and economical assay method to determine the collagenase activity is unaddressed.

6. Classification of microbial collagenases

True microbial collagenases, especially bacterial collagenases are described as enzymes that cleave helical regions of 'native' fibrillar collagen under physiological conditions[21, 41-42, 70]. However, it is frequently difficult to distinguish true microbial collagenase from gelatinases and other bacterial proteases[21]. The terms collagenolytic protease, gelatinase, collagenase, and collagenolytic enzymes establish a considerable mystification among the scientific community. It leads to the controversy and inaccuracy in both classification and nomenclature of microbial collagenases. To avoid the mystification for the use of these terms should be defined[34, 68]. As well as the frontiers should be very clear for the vertebrate collagenases and gelatinases to differentiate from microbial collagenase.

Animal collagenases split the collagen by hydrolyzing a single peptide bond across the three α-chains organized in its native triple-helical conformation. After this primary fragmentation of collagen, the attack on α-chains is very limited, and the resultant fragments tend to uncoil into collagen polypeptides, which are more susceptible to other proteases, such as gelatinases[21, 35]. At the contrary, almost all collagen types are likely to be attack by microbial collagenases at various specific sites of the α-chains[21, 50]. It is essential to stress that

a large number of microbial proteases have the capacity to hydrolyzed single-stranded and denatured collagen polypeptides. These collagenolytic bacterial proteases should not be mystified with the true microbial collagenases; that can degrade the triple-helical native collagen[21].

Matrix metalloproteinases (MMPs) is families of zinc-dependent endopeptidases (include interstitial collagenases, stromelysins, gelatinases, membrane-type metalloproteinases and others) possess collagenolytic activity[21, 35, 72]. Members of the family of MMPs are key enzymes in normal and pathological tissue remodelling. In MEROPS-the peptidase database, all MMPs belong to M10 family; however microbial collagenase belongs to M9 family with entirely different biochemical and structural properties[72]. Presently, MMPs are belongs to sub clan MA (M) of the zinc metallopeptidase (M10 family). Previously, MMPs were included in clan MB that contains only HEXXH metallopeptidases[21]. In 1999, when the three dimensional structure became available the clan MB was merged with clan MA in the MEROPS peptidase database. Presently, MMPs (M10 family) have longer zinc binding consensus sequence (HEXXHXXGXXH) as compare to microbial collagenase (M9 family) zinc binding consensus sequence (HEXXH)[21, 71-73]. Details of MMPs is presented in Table 1.

In some cases, the collagenase name was given to the different enzymes and even though that enzymes were from different families[21, 85]. *Geobacillus collagenovorans* MO-1 a thermophilic and collagen-degrading species was claimed to produce an array of collagenolytic enzymes[21, 84]. This strain produces three enzymes that are associated with the collagen breakdown. Collagenolytic protease produced by this strain was able to hydrolyze the collagen and two Pz-peptidases (metallopeptidases from peptidase family M3). As well as, it can also hydrolyze the 4-phenylazobenzyloxycarbonyl-ProLeu-Gly-Pro-D-Arg (a synthetic peptide). This collagenolytic protease is a serine protease that belongs to the

peptidase family, and the two metallopeptidases are Pz-peptidases, which only act on small oligopeptides, not on fibrillar collagens. The term collagenase is indiscriminately used to assign both metallopeptidases and serine proteases. In recent years, the remarkable progress has been made to identify new microbial collagenase and their collagen degradation mechanism. Hence, before further discussing the microbial collagenase, it is very necessary to discuss the structure and classification of microbial collagenases to avoid this confusion. A strict standard of collagenase classification should be followed in publications to prevent the controversy among the scientific community[69]. However, it is also suggested that collagenolytic bacterial proteases can be used to all proteases that are able to degrade at least one type of collagen[69]. Classification of microbial collagenase is described in Table 2 and Fig 2.

6.1. Metallocollagenases

Metallocollagenases are zinc-containing enzymes that usually required calcium ions for their optimum activity and stability. These metallocollagenases are found mostly in the vertebrates and bacteria. *C. histolyticum* and *Vibrio* sp. metallocollagenases have been widely studied[71-73]. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) are assigned these microbial collagenases (endopeptidases) to EC 3.4.24.3 group of the metalloendopeptidases sub-class (EC 3.4.24)[21]. These microbial collagenases (*Clostridium* and *Vibrio* collagenase) belongs to the MEROPS peptidase family M9[72]. The MEROPS M9 peptidase family is divided into subfamilies, M9A, and M9B[72].

6.1.1. M9A Family: *Vibrio* Collagenases

The collagenases from *Vibrio* have been widely studied after *Clostridium* collagenase. The subfamily M9A have four related groups of peptidases whose holotypes are bacterial collagenase; *Vibrio* VMC peptidase (from *V. alginolyticus* and *V. mimicus*); non-peptidase homologues and unassigned peptidases, respectively (Fig 2)[21, 71-72, 74-75]. In the first step of

collagen degradation, *Vibrio* collagenase cleaves the triple-helical collagen at a point three-quarter of the way from the *N*-terminus by hydrolyzing the preferential peptide bond X-Gly[26]. *Vibrio* collagenase cleaves native collagen with a higher degree than vertebrate metalloproteinase. *Vibrio* collagenase hydrolyzes the Pz-peptide (Pz-Pro-Leu-Gly-Pro-D-Arg) by cleaving the peptide bond Leu-Gly as degraded by *Clostridium* collagenase.

According to the substrate specificity and structural similarity to other zinc proteases, the metalloproteinases from *Vibrio* were divided into two classes: class I (enzymes cannot digest collagens) and II (can hydrolyze the collagens)[21, 76]. Based on the amino acid sequences, enzyme molecular masses, substrate specificities, and function, these two classes of *Vibrio* extracellular metalloproteinases were re-organized into three categories[76]. The molecular weight of class I enzymes is approximately 36-38 kDa, and they cannot digest the collagen. The class I enzymes are members of the M4 family (Thermolysin family). Class II enzymes are about 62-71 kDa molecular mass. These enzymes cannot hydrolyze casein but can digest collagen. Class III has ~89 kDa molecular weight. These enzymes can digest caseins, gelatin, and collagen[76]. Class II and class III enzymes are members of the subfamily M9A in the MEROPS database. However, Class II and Class III enzymes have significant differences in their structure and function. Especially, class II enzymes have a zinc-binding motif and contain no C-terminal domain. While the class III proteases have a zinc-binding motif, contain a polycystic kidney disease (PKD) like domains and a pre-peptidases C-terminal (PPC) domain at their carboxyl terminus[21, 69, 76-77]. Although, class II enzymes do not contain a C-terminal extension, it is reported that the motif in the carboxyl terminus of *V. mimicus* collagenase is involved in binding to collagen[75]. In contrast, neither the PPC domain nor PKD-like domain of class III enzymes is yet demonstrated to function as a collagen binding domain[69]. Only *Vibrio* metalloproteinase from class III (MEROPS M09.001) and class II (MEROPS M09.004) and should be considered as collagenases[21]. To best of our

knowledge, the crystal structure of *Vibrio* collagenase or its domain is not resolved. Therefore, it is still unclear that how *Vibrio* collagenase recognizes and cleaves the native collagen.

6.1.2. M9B Family: *Clostridium* collagenases

Clostridium collagenase was the first bacterial collagenase discovered and reported by Maschmann in 1937. Followed by *C. histolyticum* collagenases have been extensively studied by various research groups in basic science laboratories for medical treatment and other purposes. M9B subfamily *Clostridium* collagenase is further classified into two well-recognized classes *viz*. Class I and class II collagenases. The class I and II collagenases were differentiated on the basis of their kinetic and structural characteristics, which includes the relative activities towards different substrates (collagen, gelatin, synthetic substrates, etc.), freeze-thawing stability, and primary, secondary and tertiary structures[21]. The class I microbial collagenases are highly active on native collagen and moderately active on synthetic collagen peptides. However, class II collagenase shows moderate activity towards the native collagen and high activity towards the synthetic peptides. Matsushita's research group and his collaborators showed the presence of two collagenase genes, colG, and colH, in *C. histolyticum* chromosome. Class I collagenases being encoded by gene colG and class II by colH[55, 69, 78-80]. In addition to colG and colH, collagenases gene from other *Clostridium* species are also reported, such as colA from *C. perfringens* and colT from *C. tetani*. In MEROPS database, all *Clostridium* collagenases are members of the M9 family[69, 71-72]. More investigations on microbial collagenases and their encoding genes are necessary to draw a conclusive evolutionary picture for these enzymes.

A *Clostridium* collagenase molecule (unit) is composed of the *N*-terminal activator (collagenase) domain and the C-terminal recruitment (peptidase) domains that contain a conserved zinc-binding motif and function as a catalytic domain. The recruitment domains

usually contain one or two collagen binding domains (CBDs) and one or two polycystic kidney disease (PKD)-like domain(s). The recruitment domains are not required for degradation of triple helical collagen, but most likely needed for larger collagen entities such as fibrils. Mostly, the activator domain and catalytic domain in the collagenase module remain closed during native collagen cleavage but relax to the open ground state once the native collagen is degraded[38]. The *Clostridium* collagenases hydrolyzed native collagens into a mixture of smaller fragments or peptides (Fig 3). The distinct hyper-reactive sites for class I and II *Clostridium* collagenases are Y-Gly bonds in the repeating Gly-X-Y collagen sequence. The two class of collagenases bind to different portions of collagen and have different specificities to cut native collagens, i.e. showing synergy in collagen degradation[69, 80-81, 83-84].

6.2. Serine collagenolytic proteases and serine collagenases

Several S8 proteases with collagenolytic activity have been reported from bacteria, archaea, crab, crustaceans, fish and human pathogens[36, 69, 88-89]. Some S8 proteases have been reported in recent years to be collagenolytic proteases. Proteases of this family (S8 family) are characterized by an Asp/His/Ser catalytic triad and an alpha/beta fold catalytic center containing a seven-stranded parallel beta-sheet[69]. Mostly proteases in the S8 family have no collagenolytic activity. Serine collagenolytic proteases sometimes referred as serine collagenases. Serine collagenases reported till now possess broad proteolytic activities[21, 69, 71-73]. The thermostable serine collagenolytic proteases protease from *Geobacillus collagenovorans* MO-1 is the first reported S8 collagenolytic protease. This enzyme has a C-terminal collagen-binding domain, and its cleavage sites on collagen are various but specific[86-87]. Recently, two S8 proteases from marine bacteria, *Pseudoalteromonas* sp. were characterized as collagenolytic proteases, and their actions toward collagen degradation were also revealed[69, 88-93]. However, there is no evidence that serine proteases can hydrolyze the

triple-helical native collagen. Therefore, it is suggested that these peptidases could be considered as serine gelatinases, not as serine collagenases[21].

7. Fermentative production of collagenase

A limited number of reports are available on microbial collagenases production. Most of the microbial collagenases reported so far have been produced in submerged fermentation (SmF) process[8-10, 45-46]. Various types of collagen supplement (native collagen, gelatin/collagen hydrolysate, etc.) have been used in the culture media for the production of extracellular collagenase enzymes. Various researchers used the malt extract with 1% gelatin and soybean flour as a supplement for culture media, which can induce the collagenase enzyme production[8-10, 46, 58]. Substrates such as insoluble collagen[34, 51] and fish collagen[27] have also been reported as inducers in collagenase production. Crude substrate such as fish scale powder, fish skin, mammalian, shrimp and crab by-products also used as sole carbon/nitrogen sources in culture media for collagenase production by some research group[27, 29, 34, 51]. A maximum collagenase activity of 240 U/mL from *Nocardiopsis dassonvillei* in SmF using shrimp and crab by-products as the sole nitrogen and carbon source have been reported[29]. The extracellular collagenase produced by *Bacillus pumilus* showed the maximum 129.5 U of collagenase activity[44]. The collagenase production from *B. cereus* (23.07 U/mL) and *Klebsiella pneumoniae* (10.84 U/mL) are also been reported[52]. Tran and Nagano reported a maximum activity of 3.07 U/mL in the collagenase from *B. subtilis*[60]. A maximum collagenolytic activity of 6.8±0.4 U was reported from *Candida albicans* collagenase[46]. The potential use of microbial collagenase and their high demand attracting the interest of the scientific community to find new microbial species/strains those are able to produce high titers of extracellular collagenases with novel properties.

8. Strategies used to improve microbial collagenase production

In the last decade, collagenase research has gained momentum because of additional therapeutic, industrial and biotechnological applications other than those of conventional proteases[94-95]. The development of the low-cost industrial media formulations for production of microbial collagenase can play a significant role in the cost of product, concentration, yield, and productivity. Media composition is one of the most significant factors in the industrial production of collagenase because 30-40% of the production costs are due to the high-cost growth media[9-10]. Optimizing the cost/benefits ratio of such processes is, therefore, a leading concern. The cost of collagenase production is a major hurdle in successful industrial applications. An inexpensive medium can be used for the economically feasible, industrial collagenase production with higher yield. Optimization of physiochemical conditions (pH, temperature, carbon source, and gelatin, etc.) for collagenase production from *B. cereus* CNA1 and *K. pneumoniae* CNL3 were reported[52].

Statistical design methods

To meet the commercial requirement, optimization of different process parameters and media composition are well known methods for the over production of enzymes in large quantities [94-98]. Culture media formulation and physicochemical bioprocess conditions are reported as significant factors to enhance the microbial collagenase production[96]. However, designing the medium is a laborious, expensive and often time-consuming process involving several experiments. Statistical tools such as response surface methodology (RSM) play an important role to optimize the level of various bioprocess variables for enhanced production of extracellular microbial collagenases[9-10, 94-95]. Statistical optimizations of microbial collagenase production under SmF process has been reported[9-10, 46].Using the factorial design and RSM approachs, optimization of culture conditions (pH, time, temperature, inoculum size, orbital agitation speed and substrate concentration) for extracellular collagenase production from *C. albicans* URM3622 were studied using 2^{6-2} factionary factorial and 2^3 full

factorial designs[46]. A full two-level factorial design with three variables (medium pH, temperature and soybean flour concentration) followed by a central composite design (CCD) was reported for optimization of collagenase production from *Penicillium aurantiogriseum* URM4622[9-10]. They observed an improvement of 7.06U higher collagenolytic activity from *C. albicans* URM3622[46] and 5-fold increase activity from *P. aurantiogriseum* URM4622[9-10] after statistical experimental designs. However, an improve in microbial collagenase production requires a comprehensive understanding of microbial strain, metabolic functions through metabolic model construction, and following *in silico* experimentation using systems biology methods[132]. These *in silico* experiment can suggest the cell manipulations that can also be applied using *in vitro* synthetic biology techniques, leading to increased microbial collagenases production[132].

9. Gene cloning of collagenase

Microbial collagenases have been purified from a limited number of bacterial species, and their corresponding genes have been cloned and sequenced[24, 38, 50, 55-57, 65, 83, 102-104]. The expression of *Burkholderia pseudomallei* putative collagenase in *Escherichia coli* was reported[105]. They cloned the collagenase gene, and the protein was expressed as a glutathione S-transferase (GST) fusion and purified. Though, there is a lack of information about the domain composition and features of *B. pseudomallei* collagenase. However, collagenase activity with FALGPA peptide was weak, but this could be related to the glutathione S-transferase fusion[21]. A high gelatinase activity was detected which led the authors to postulate that *B. pseudomallei* express an active collagenase/gelatinase, but no class assignment was possible[105]. The catalytic domain of collagenase G from the *C. histolyticum* has been cloned[38], recombinant and expressed in *E. coli* and purified using the affinity and size-exclusion column chromatographic methods[83]. Ducka et al. established an *E. coli* expression system for a range of constructs of collagenase T from the *C. tetani* and collagenase G and H

from *C. histolyticum*[54, 101]. The collagenase gene was cloned from *Grimontia* (*Vibrio*) *hollisae*, and its complete nucleotide sequence was determined[57]. *Brevibacillus* expression system produced the recombinant collagenase. Collagenases, or genes encoding collagenases, have been identified in many *Clostridium* species: *C. histolyticum, C. perfringens, C. botulinum, C. tetani and C. difficile* [21, 55, 69]. The cloning of the collagenase G and H genes simplified the development of microbial collagenase expression systems. The *Cl. perfringens* expression system alleviated the translational problems and expressed ColH[28]. Mutagenization and nucleotide sequence analysis of a cloned gene would provide insights into some structure-function relationships for the enzyme. Matsushita et al. cloned and sequenced the col gene encoding a 120 kDa collagenase from *C. perfringens*[55-56]. They also described the cloning and sequencing of the colH gene encoding 116 kDa collagenase from *C. histolyticum*. It is evident that a lot of microbial collagenases are waiting for the deep structure, classification and characterizations[105].

10. Purification of microbial collagenases

Purification of microbial collagenase has been complicated due to the presence of multiple forms and other proteases with similar physical and chemical characteristics[55]. The purified form of collagenase is required to study the biochemical properties, enzyme structure, catalytic mechanism, structure-function relationships and their biotechnological and medical applications[96]. However, the commercial applications of collagenases in food and leather industries do not require a highly purified enzyme. Purification of microbial collagenase by various techniques resulted in the identification of two to seven different microbial collagenase fractions[55-56]. Microbial collagenases have been purified by few researchers[36, 41-44, 48, 55, 65-66, 77, 83, 88, 99-100]. The purification of microbial collagenase from *C. histolyticum*[38], *B. pumilus*[44], *B. licheniformis*[27] and *Rhizoctonia solani*[51] have been reported. Various procedures (ammonium sulfate precipitation, ultrafiltration, immobilized metal

affinity chromatography, amylose affinity chromatography, gel filtration chromatography, ion exchange chromatography, size exclusion chromatography and removal of *N*-terminal tag) have been applied for the purification of microbial collagenases[2, 38, 40, 50-51, 81-83]. Despite, several research groups did extensive studies, there is no consensus on the number of enzymes and their properties. This may be due to the difference in procedures used for the enzyme preparation and strains used for collagenase production. However, most of the report on the microbial collagenase purification schemes carried out at small/laboratory scale. Though, the excessive purification is very expensive and also reduces overall recovery of the microbial collagenase. 101-fold purification of a thermostable alkaline collagenase from *Thermoactinomyces* sp. was reported[40]. The purified *B. licheniformis* and *B. pumilus* collagenase showed a 26.3-fold and 31.53-fold increase in the specific activity, respectively[27, 44]. Purification of recombinant collagenase from *Grimontia (Vibrio) hollisae*[57], *C. histolyticum* (ColH) 116 kDa collagenase[133], and *C. perfringens* 120 kDa collagenase[55] have been reported.

11. Characterization of microbial collagenase

A limited number of purified microbial collagenases have been characterized by their activity and stability profiles corresponding to temperature, pH, effects of metal ions and chelating agents. The biochemical characteristics of microbial collagenase were given in Table 3. The properties of collagenase depend on types of the microbial species/strains. Most of the collagenases have a high apparent molecular mass within the range of 50-120 kDa. Very few reports are available on the effect of pH on collagenase activity. The optimum pH of microbial collagenase is found to be in the range of 5 to 9.5. The optimum temperature for the activity of microbial collagenase may be associated with temperature required for the growth of microorganism as well as types of microbial species/strains[96] and found to be in the range of 40-65 °C. Ions play a significant role to improve the activity of microbial

collagenase. Structural stability and activity of *Clostridium* collagenase were enhanced in the presence of calcium ions[28, 101]. However, collagenase activity was majorly inhibited by Fe^{2+}. Effects of different metal ions on the activity of microbial collagenase were summarized in Table 4.

12. Applications of microbial collagenases

The applications of microbial collagenases are wide, including areas such as food, tannery, meat, cosmetic industries, production of pharmaceutical compounds, and bio-restoration of frescoes, etc.[21, 35, 69] (Fig 4). Microbial collagenases are also applied for the isolation and cultivation of mammalian cells. Therefore, it has a significant role in the medical sectors. It can be used to treat burns, wound, scar tissue, transplantation of specific organs, peyronie's disease, destructive fibrosis, liver cirrhosis and to clean the blood for improve screening in medical diagnostics[21, 32].

12.1. In food processing and allied industries

Microbial collagenase can be used in several food applications (Fig. 5). However, the commercial use of microbial collagenase in the food industry is limited to few applications due to its higher cost.

In meat tenderization

Tenderness is one of the most important sensory qualities of meat, which influenced by the length of sarcomeres, the integrity of connective tissue (background toughness) and myofibrils (actomyosin toughness)[106-108, 113]. Toughness is one of the most significant causes of unacceptability in meat quality[106]. In developing country like India, most of the animals are reared for dual purpose and slaughtered only after the end of their productive economic life (useless for any other purpose). The meat obtained from these old/spent animals is very tough. Therefore, tenderization process is required to increase the consumer acceptability of raw meat and meat products.

To enhance the meat quality, the tenderization problem can be effectively overcome using the enzymatic tools and process modifications[109]. Treatment by proteases (microbial and plant origin) is one of the most progressive method used to increase the meat tenderness. These proteases have broad specificities toward meat proteins. Therefore, microbial/plant protease might result in several undesirable sensory attributes in tenderized meat. Collagen (which accounts for 80% of the connective tissue) is also responsible for the toughness of red meats, and its digestion induces the meat tenderization process. Thus, microbial collagenase that showed the specific hydrolyzing activity towards collagen as compared to other proteins would be more advantageous[111]. Therefore, microbial collagenases have been proposed to be an attractive alternative to non-specific plant/microbial proteases in meat tenderization process[110]. Collagenases from *C. histolyticum* and *Vibrio* B-30 have shown promising results in the meat tenderizing studies[110, 112, 127]. However, concerns regarding safety issues such as pathogenicity and other unfavorable effects have limited the industrial use of microbial collagenases from these organisms in the process of meat tenderization. An ideal meat tenderizer should be a specific enzyme with high activity at the room temperature and should be easily inactivated during the cooking process of meat/meat products[113]. Microbial collagenase from non-pathogenic and safe microorganisms with an improved specificity toward collagens can be used as an ideal meat tenderizer. Zhao et al. demonstrated that a cold-adapted collagenolytic enzyme from *Pseudoalteromonas* sp. SM9913 has significantly reduced the beef meat shear force and kept the fresh color and moisture as compared to commercially used papain and bromelain. The collagenolytic enzyme had a strong selectivity for the degradation of collagen/myofibrils proteins and had a distinct tenderization mechanism[113].

In collagen extraction

Collagen and its hydrolyzed form gelatin are widely used in the food industries. Traditionally, collagen is extracted using an acidic solution with or without an aid of an enzyme[4]. The yield of collagen extraction can be increased by using low concentrations of the microbial collagenases[52]. Microbial collagenases facilitated the collagen extraction via the cleavage of telopeptide region[52]. A combination of collagenases from *B. cereus* and *K. pneumoniae* strains with acid treatment yielded a higher collagen recovery from salmon skin preparations as compared to using an acid treatment alone[52]. Therefore, the microbial collagenase might be used for the extraction of collagen for industrial applications.

In preparation of bioactive collagen hydrolysate and collagen peptides

In publications, the "collagen hydrolysate" is defined by several terms such as "collagen peptides", "collagen degradation products", "hydrolyzed collagen", and "hydrolyzed gelatin" to designate the same product[116]. Collagen hydrolysate is usually prepared from gelatin using proteolytic treatment. Proteases such as trypsin, chymotrypsin, pepsin, alcalase, collagenase, bromelain and papain are the most regularly used enzymes for collagen hydrolysate preparation. The average molecular weight of commercially available collagen hydrolysate ranges between 0.5 to 20 kDa[7, 114-116]. The structure, composition, molecular weight distribution and functional properties of collagen hydrolysate depend on the processing conditions, raw material as well as the specificity of the enzyme used to hydrolyze the gelatin. Collagen hydrolysate has been approved as Generally Recognized as Safe (GRAS) by the Center for Food Safety and Nutrition, US Food and Drug Administration (USFDA)[7]. From a nutritional point of view, collagen hydrolysates are well acknowledged for their safety[7]. Therefore, collagen hydrolysates/peptides derived from animal/seafood based collagen is of great interest as a potential ingredient in functional food and beverage products[7].

Collagen hydrolysate (collagen-derived ingredients) are widely used in food, cosmetic and pharmaceutical industries due to their gelling capacity, texturizing, thickening, and water binding capacities. As well as their swelling and solubility properties, emulsion, foam formation and stabilization, adhesion and cohesion, protective colloid function and film forming capacity[7, 116]. It is commonly used as a dietary supplement or included in various foodstuffs. The ingestion of collagen peptides has been shown to induce numerous biological processes[114-119]. Some clinical studies reported that daily oral intake of collagen hydrolysate decreased joint pain, reduced the skin wrinkles and improved skin properties. Other studies have also suggested that a hydrolysate enriched diet can improve bone collagen metabolism[7]. Collagen hydrolysate can be used as a relevant alternative in the design of future nutritional approaches to managing osteoarthritis and osteoporosis prevention[116]. Collagen hydrolysate/collagen peptides have exhibited strong antioxidant, anti-fatigue, antimicrobial and angiotensin-converting-enzyme (ACE) inhibitory activities[8, 128].

Microbial collagenases are also used as hydrolyzing agents for the preparation of collagen hydrolysates/collagen peptides. Preparations of strong antimicrobial and radical scavenging properties containing bovine collagen hydrolysates were reported using *P. aurantiogriseum* URM 4622 collagenase[8]. Ding et al.[128] were demonstrated an *in vivo* anti-fatigue activity and antioxidant activity of jellyfish collagen hydrolysate. Specific antifreeze peptides derived from shark skin collagen hydrolysate have been reported[131]. Guo et al.[129] isolated Alaska Pollock skin collagen-derived mineral chelating peptides that may have the potential applications as functional food ingredient in the management of mineral deficiencies. Squid skin collagen hydrolysates have anti-hyaluronidase, antityrosinase, and antioxidant activites[130].

In meat and seafood processing byproducts valorization

Microbial collagenase may also be used to recover biomolecules/biopolymers such as collagen, gelatin, collagen peptides, collagen hydrolysate, protein hydrolysates, etc. from different animal/seafood processing by-products[139]. The cost-effective production of collagenase from *B. tequilensis* was achieved by utilizing meat industry wastes as the sole nitrogen and carbon source. However, the decrease in extracellular collagenase production from *B. tequilensis* can be negotiated by utilization of waste animal skin for useful product synthesis and waste management[34].

12.2. In leather processing

Due to water pollution, careless solid wastes disposal and gaseous emissions, leather industries are categorized as a red industry and are under deep pressure to develop the environmentally efficient leather-making processes to meet the terms of modern pollution and discharge legislation. Conventional leather processing involves the use of a large number of different chemicals such as lime[135]. Due to global concerns about the environmental impact of leather industry have led tanners to reduce the elements of toxicity, organic and inorganic chemicals in the effluents[136]. Therefore, it is a big challenge today to find out the environmental-friendly alternatives to chemical processing of hides and leather. In this respect, developments of enzymatic processes as alternatives to some of the chemical-based processing are being encouraged because they not only yield quality improved products but also reduce the use of hazardous and polluting chemicals[135-137].

Collagenases also have potential application in leather industry[21, 32]. They are used as biocatalysts to improve the dye exhaustion process. Dyeing is an important process of leather industry. Synthetic colorants that are used in leather dyeing are also a major source of environmental pollution. The unexhausted dyes present in the leather industry effluents are frequently resistant to bioremediation, which is a major concern[120]. After leather tanning, use of bacterial collagenases results in the opening-up of the fibrous leather network. In the

leather matrix, diffusion of dyes can be enhancd by opening-up of the fibrous collagen network. This process results in an uptake of dye up to 99% using this eco-friendly approach and also improved the bulk properties such as softness, fullness, grain smoothness, feel and general appearance of the leather[121].

Dehairing is the first step in leather making process. In leather industry, the dehairing process creates a high level environmental pollution. Therefore, the enzymatic dehairing process may help to reduce the pollution load and collagen damage. A high degree of control over the process is the major limitation of enzymatic dehairing process[122]. It has been proposed the use of collagenase enzymatic formulations for dehairing process[123]. However, the efficacy of the collagenase and their cost are restricting their commercial applications in dehairing processes[123].

12.3. In laboratory as an experimental reagent

In the laboratory, microbial collagenases are widely used as experimental reagents in the laboratory-scale studies such as to isolated rat liver cells and scission of collagen-like peptides infusion proteins[35, 58]. Cell culture has been widely used as an essential tool in biotechnology, molecular biology, and toxicology, to address the important economic, technical and scientific issues in biology. There are various types of cultured cells, such as primary cultures, cell lines, and cell strains, in which different types of enzymes are frequently used for tissue disaggregation. Microbial collagenase formulation has been successfully used for the isolation of cells from bone, endothelial cells, neuronal cells, and isolation of the islets of Langerhans, among others[21]. The fibrous tissues with high collagen content are particularly resistant to trypsin digestion. Microbial collagenases are especially valuable in the case of fibrous or sensitive tissues in which trypsin use would be ineffective or damaging[25]. Furthermore, the *Clostridial* collagenases in association with other enzymes

have been increasingly applied for numerous medical proposes. For example, the creation of autologous dermal tissue from fibroblasts was isolated using *Clostridium* collagenase[21, 25].

12.4. In human health and nutrition

One of the most significant applications of microbial collagenase is found in the medical industries. Collagen constitutes one-third of the body protein in humans, reflecting its extraordinary role in health and disease[4, 38]. Microbial collagenases have directly been employed in clinical therapy (debridement of burns, wound healing, sciatica, retained placenta, lumbar disc herniation, chronic total occlusions treatment and to enhance adenovirus-mediated cancer genetic therapy or electro-genetic therapy)[9, 21]. The use of *Clostridium* collagenase is considered as a capital addition in wound debridement, helping to avoid the surgery complications and also limiting the progress and enlargement of the necrotic tissue[21, 124-125]. *Clostridium* collagenase is also applied for treatment of Dupuytren's' disease (DD)[21]. Enzymatic fasciotomy with *Clostridial* collagenase targets excessive collagen deposition and ruptures the fibrous cords that cause contractures in DD. Historically, treatment options for Dupuytren's contracture were limited to surgical procedures. The FDA approved *Clostridium* collagenase to DD treatment[21]. Microbial collagenases are used various types of destructive fibrosis. The recent application of microbial collagenases is related to the genetic therapy. The administration of the naked nucleic acids into the cells and tissues can be considered as the simplest and safest method for gene delivery[21]. The application of microbial collagenase (with hyaluronidase) increases the transfection efficiency in tumors, without promoting the spreading of metastases[126].

12.5. In environmental protection

Besides the pathogenic microorganisms, various collagenase-producing microorganisms have been isolated from the marine and terrestrial sources. Although a huge quantity of collagen is produced annually in the aquatic/marine biosphere alone, there is no

substantial accumulation of collagen in ocean sediments because they are biodegraded by the naturally occurring bioconversion process mediated by collagenolytic marine microrganimsms[69]. Zhang et al. revealed that the mechanisms of environmental microorganisms on collagen degradation are helpful for the study of the global nitrogen cycle[69]. Furthermore, the role of *Clostridium* collagenases in the putrefaction process of the carcass is well documented.

13. Challenges associated with microbial collagenases in food and nutrition industry

The market share of microbial collagenase considered higher as compared to the proportion of animal and plant collagenase. Microbial collagenase (*Clostridium* collagenase) properties are well documented and widely used in medical sectors. However, the study of collagenase-producing microorganisms and their importance in food and nutrition are scanty. Major challenges and limiting factors for microbial collagenase applications in the food and nutrition sector are discussed.

Sources of collagenase-producing microorganisms: The ecosystem is having a vast abundance of microorganisms. The microorganisms involved in the degradation of animal waste/by-products may be able to produce the extracellular collagenase. Marine microorganisms have recently emerged as excellent sources of collagenase. The most of the commercially available and well-studied collagenase-producing microorganisms are anaerobic and pathogenic. The collagenase produced from pathogenic microorganism creates the specific concerns about the pathogenicity of the enzyme. Presently, a very few manufacturers are involved in the production of microbial collagenase (*Clostridium* collagenases), globally. Due to the higher cost and safety concerns of collagenase, their potential application in food and nutrition sectors is scanty. Therefore, in the view of the food and nutrition applications, there is a vast requirement to search the non-pathogenic, extracellular collagenase-producing microorganisms.

Screening, optimization and production of collagenase: A lot of controversies were found to screen the collagenase-producing microorganisms among the scientific literature, due to low denaturation temperature (~30-37 ^{o}C) and acidic solubility of the collagen. Further, a simple, cost-effective and high throughput method for determination of collagenase activity is still underway. However, radiolabeled and synthetic peptides used to determine the collagenase activity are very laborious, time-consuming and expensive. The cost-effective collagenase production is a critical challenge to the scientific community. The sustainable collagenase production for food and nutrition sector is becoming a priority due to the current dependency upon *Clostridium* collagenase. The use of cheaper agro-industrial by-products as fermentation medium can also help to reduce the enzyme cost. The optimization of processes parameters (medium composition nutrients and culture conditions) for collagenase production is very scanty. It is a critical challenge to define the level of each variable (physico-chemical, culture conditions, medium composition, etc.), and their possible interactions between the variables. Collagenases producing microbial strains can be typically designed using combinations of gene manipulations (addition and removal of genes). This process may afford the potential to the successful production of microbial collagenase. The challenges to improving the production of collagenase may also be achieved using synthetic and systems biology.

Classification, crystal structure and collagen degradation mechanism of microbial collagenase: A new overview to classify and purify the microbial collagenase is needed for the identification of true microbial collagenase from the similar other collagenolytic proteases. Still, there are no specific recommendations for the purification and classification of the microbial collagenase. The mechanism of microbial collagenase for the degradation of native collagen is still unclear. To best of our knowledge, no crystal structure of a microbial collagenase has been reported except *Clostridium* collagenase. Hence, the microbial

collagenase classification, purification, crystal structure and their collagen degradation mechanism are major challenging task for scientific communities. Microbial collagenase can also be used for the production of novel true collagen peptides (native collagen peptides). These collagen peptides were reported for their health promoting activities. Nowadays, the development of true/ native collagen peptides and their potential applications as functional food ingredients may create the opportunities for their diverse applications in food and nutrition industry.

14. Directions and recommendations for future research

Potential applications studies of microbial collagenase are not well existed and evolving in the direction of food, nutrition, and other related industrial applications. Currently, very few studies on the fermentative production and application of microbial collagenase were reported[8-10, 45, 46]. There is a huge controversy about proper and unambiguous identification and classification of collagenase-producing microorganisms. To reduce the collagenase cost, we have to look for non-pathogenic microbial sources for the collagenase production using cheap agroindustrial by-products. The collagenase production from non-pathogenic strains can increase the food industries application by avoiding the pathogenicity and safety concerns. Most of the reported microbial collagenases have not been structurally or enzymatically characterized. Therefore, the exploration of the specificity, structure, and functions of microbial collagenase could provide a further understanding of collagen degradation mechanism and also uncover for various potential biotechnological applications.

It would be interesting to study the of microbial collagenase role to improve the quality of food, meat, and other related value-added products. Future research and studies should be focused on multidisciplinary areas such as the production of bioactive collagen peptides, collagen hydrolysate, and development of novel functional food and beverage

ingredients. Furthermore, it will also be important in the extraction of collagen and their application in food and nutrition industries. Hence, studies on the microbial collagenase production and applications would be interesting, rewarding and beneficial to improve the growth of the food, nutrition, and other allied sectors.

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Competing interests

The authors declare that we have no conflict of interest.

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Captions for figures

Fig 1 Major collagenases and different sources of microbial collagenases

Fig 2 Schematic classification and domain organization of microbial collagenases. A) The domain structures of collagenase from *Vibrio parahaemolyticus* (a); *V. mimicus* (b); *V. alginolyticus* (c); Col G from *Clostridium histolyticum* (d) and Col H from *C. histolyticum* (e).

AD = Activator domain; CD = Catalytic domain (Peptidase domain); PKD = Polycystic kidney disease like domains; CBD = Collagen binding domains; PPC = Pre-peptidase C terminal domains

Fig 3 A schematic model for the degradation of native collagen by the microbial collagenase

Fig 4 Applications of microbial collagenases in various sectors

Fig 5 Possible applications of microbial collagenases in the food and nutrition

Table 1. Details of matrix metalloproteinases (MMPs) with MEROPS the peptidase database ID (Source: Duarte[21]; Rawlings[71-73])

MMP	Other name	EC Number	Substrate	MEROPS ID
Collagenases				
MMP-1	Collagenase 1, interstitial collagenase, matrix metalloproteinase 1, vertebrate collagenase, fibroblast collagenase	3.4.24.7	Type I, II, III, VII, VIII, IX and X fibrillar collagens, gelatin, aggrecan, fibronectin	M10.001
MMP-8	Collagenase-2, neutrophil collagenase	3.4.24.34		M10.002
MMP-13	Collagenase-3, AgMMP 3, rat collagenase	NA		M10.013
MMP-18	Xenopus collagenase, collagenase-4, xCol4	NA		M10.018
Gelatinases				
MMP-2	Gelatinase A, 72 kDa gelatinase, type IV Collagenase,3/4 collagenase, matrix metalloproteinase 5, tissue gelatinase	3.4.24.24	Nonfibrillar collagens, gelatin, type IV, V, VII, X, XI and XIV collagen, elastin, aggrecan, fibronectin, galectin-3	M10.003
MMP-9	Gelatinase B, type IV	3.4.24.35		M10.004

	collagenase, type V collagenase, 92 kDa gelatinase, macrophage gelatinase, neutrophil gelatinase			
Stromelysins				
MMP-3	Stromelysin-1, transin, proteoglycanase, collagenase activating protein, matrix metalloproteinase 6, procollagenase activator	3.4.24.17	Proteoglycans, collagen telopeptides, fibronectin, laminin, casein, gelatin, type III, IV, V, VII, IX, X and XI collagens	M10.005
MMP-10	Stromelysin-2, transin-2	3.4.24.22	collagen, elastin,	M10.006
MMP-11	Stromelysin-3	NA	fibrinogen, laminin,	M10.007
MMP-12	Metalloelastase, macrophage elastase	3.4.24.65	elastin	M10.009
Matrilysins				
MMP-7	Matrilysin-1, putative metalloprotease, putative metalloproteinase-1 (PUMP-1), uterine metalloendopeptidase	3.4.24.23	Fibronectin, laminin, type IV collagen, proteoglycans, , gelatin, elastin, fibrinogen, laminin, aggrecan, pro-MMP-1, pro-MMP-2, pro-MMP-7, pro-MMP-8, and pro-MMP-9	M10.008
MMP-26	Matrilysin-2, endometase	NA		M10.029

Membrane-type				
MMP-14	MT1-MMP, MT-MMP-1, membrane-type matrix metalloproteinase 1, matrix metalloproteinase, membrane-type 1	3.4.24.80		M10.014
MMP-15	MT2-MMP, MT-MMP-2, membrane-type matrix metalloproteinase 2, matrix metalloproteinase, membrane-type 2, SMCP-2	NA	Large tenascin-C, fibronectin, laminin	M10.015
MMP-16	MT3-MMP, MT-MMP-3, membrane-type matrix metalloproteinase 3, matrix metalloproteinase, membrane-type 3, ovary metalloproteinase, membrane-type matrix metalloproteinase (*Gallus domesticus*)	NA		M10.016
MMP-17	MT4-MMP, MT-MMP-4, membrane-type matrix metalloproteinase 4, matrix metalloproteinase,	NA		M10.017

	membrane-type 4			
MMP-24	MT5-MMP, MT-MMP-5, membrane-type matrix metalloproteinase 5	NA		M10.023
MMP-25	MT6-MMP, leukolysin, MT-MMP-6, membrane-type matrix metalloproteinase 6, MT-6 MMP	NA		M10.024
Other MMPs				
MMP-19	Matrix metalloproteinase 19, RASI-1, RASI-6	NA		M10.021
MMP-20	Enamelysin	NA	Type IV, V collagens, fibrin, elastin, gelatin, fibronectin, casein, laminin, aggrecan, Amelogenin, fibrinogen, α1-antitrypsin	M10.019
MMP-21	XMMP (Xenopus)	NA		M10.026
MMP-23	Cysteine-array matrix matalloproteinase, CA-MMP, femalysin	NA		M10.022
MMP-27	CMMP (*Gallus domesticus*), matrix metalloproteinase 22, MMP-22 (*Gallus domesticus*), MMP-27 (*Homo sapiens*), matrix metallopeptidase 27	NA		M10.027
MMP-28	Epilysin	NA		M10.030
-	Microbial collagenase	3.4.24.3	Native collagen	M09.001

	M09.002
	M09.003
	M09.004

NA = not assigned

Table 2 Characteristics of representative of microbial collagenases according to MEROPS peptidases database[72]

Peptidases and Homologues	MEROPS ID
A. Peptidase Family M9A	
Bacterial collagenase V	M09.001
VMC peptidase	M09.004
Subfamily M9A non-peptidase homologues	non-peptidase homologue
Subfamily M9A unassigned peptidases	unassigned
B. Peptidase Family M9B	
Bacterial collagenase G/A	M09.002
Bacterial collagenase H	M09.003
Subfamily M9B non-peptidase homologues	non-peptidase homologue
Subfamily M9B unassigned peptidases	unassigned

Table 3 The biochemical characteristics of microbial collagenase

Organism Name	pH optima	Temp. optima (^{o}C)	Molecular mass (kDa)	References
Bacillus licheniformis F11.4	7	50	124	Ace Baehaki[27]
Clostridium histolyticum	ND	ND	116	Jung[133], Matsushita[134]
C. perfringens	7.2	42	120	Matsushita[66]
C. histolyticum	ND	ND	116	Yoshihara [56]
Grimontia (Vibrio) hollisae 1706B	ND	ND	84	Teramura[57]
B. subtilis FS-2	9	50	125	Nagano[43]
Penicillium aurantiogriseum URM4622	8	45	39.16	Lima[45]
Candida albicans URM3622	8.2	45	ND	Lima [46]
B. cereus CNA1	7	45	ND	Suphatharaprateep[52]
Klebsiella pneumoniae CNL3	6	40	ND	
Thermoactinomyces Sp. E-21	9.0-9.5	60-65	50	Petrova[40]
Rhizoctonia solani	5	40	66	Hamdy[51]
B. pumilus Col-J	7.5	45	58.64	Wu[44]
Nocardiopsis dassonvillei NRC2aza	8	55	ND	Abdel-Fattah[29]

ND = not determined

Table 4 Effect of different metal ions on activity of microbial collagenase

Source of enzyme	Inhibition	Activation	Reference
Clostridium histolyticum	ND	Ca^{2+}	Ohbayashi [28, 101]
C. histolyticum	ND	Zn^{2+}	Jung[133]
Bacillus licheniformis F11.4	Fe^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}	Ca^{2+}, Cu^{2+}	Ace Baehaki[27]
B. pumilus Col-J	Mn^{2+}, Pb^{2+}	Ca^{2+}, Mg^{2+}	Wu[44]
Rhizoctonia solani	Fe^{2+}, Hg	Co^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+},	Hamdy[51]
Thermoactinomyces Sp. E-21	Fe^{2+}, Cu^{2+}, Mn^{2+}, Sr^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}	Mg^{2+}, Ca^{2+}, Co^{2+}	Petrova[40]
C. perfringens	ND	Zn^{2+}, Ca^{2+}, Mg^{2+}	Matsushita[55]

ND = not determined

Fig. 1

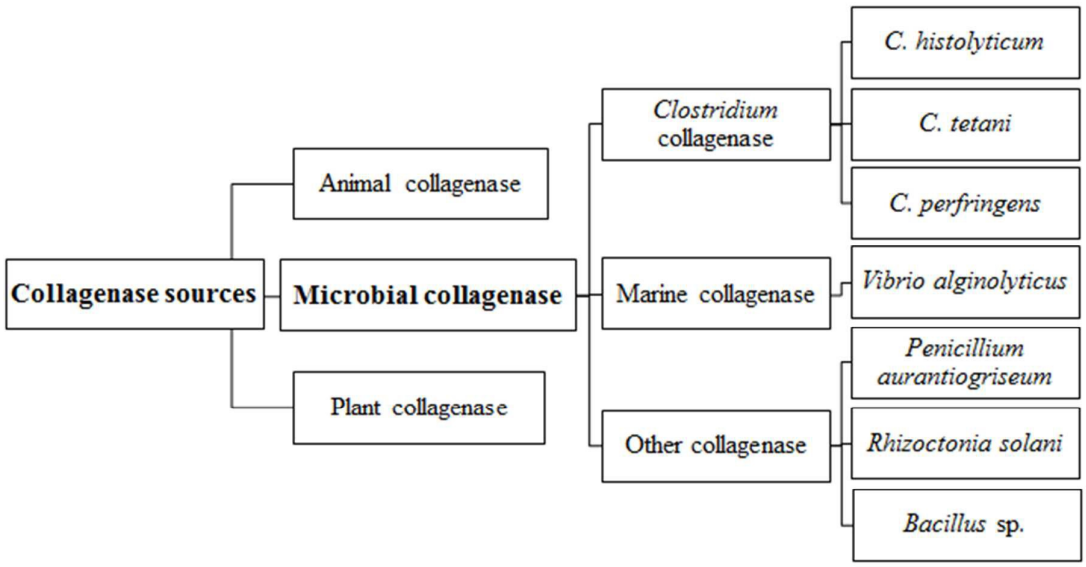


Fig. 2

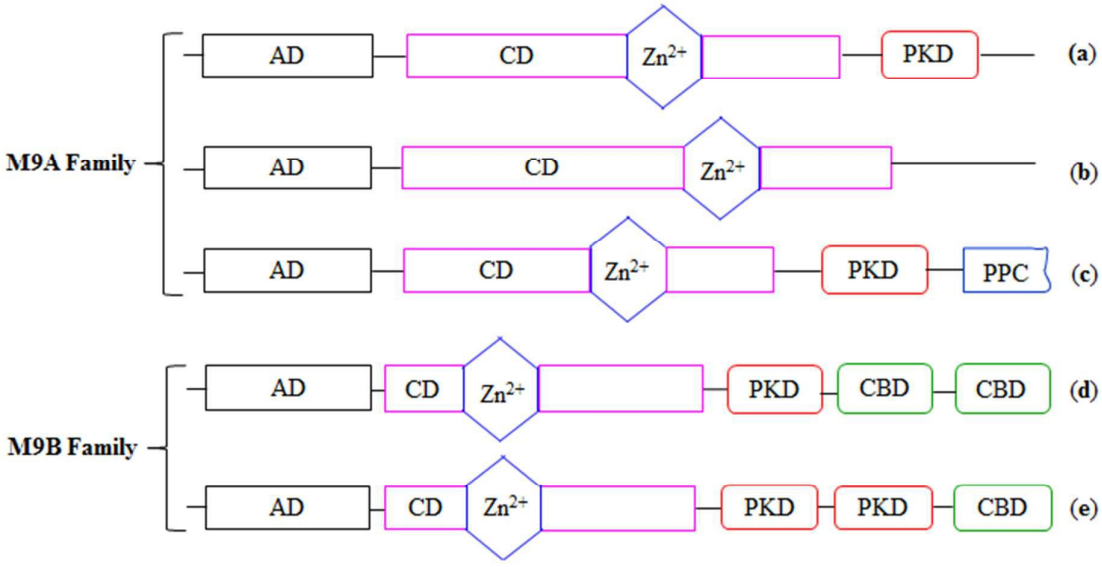


Fig. 3

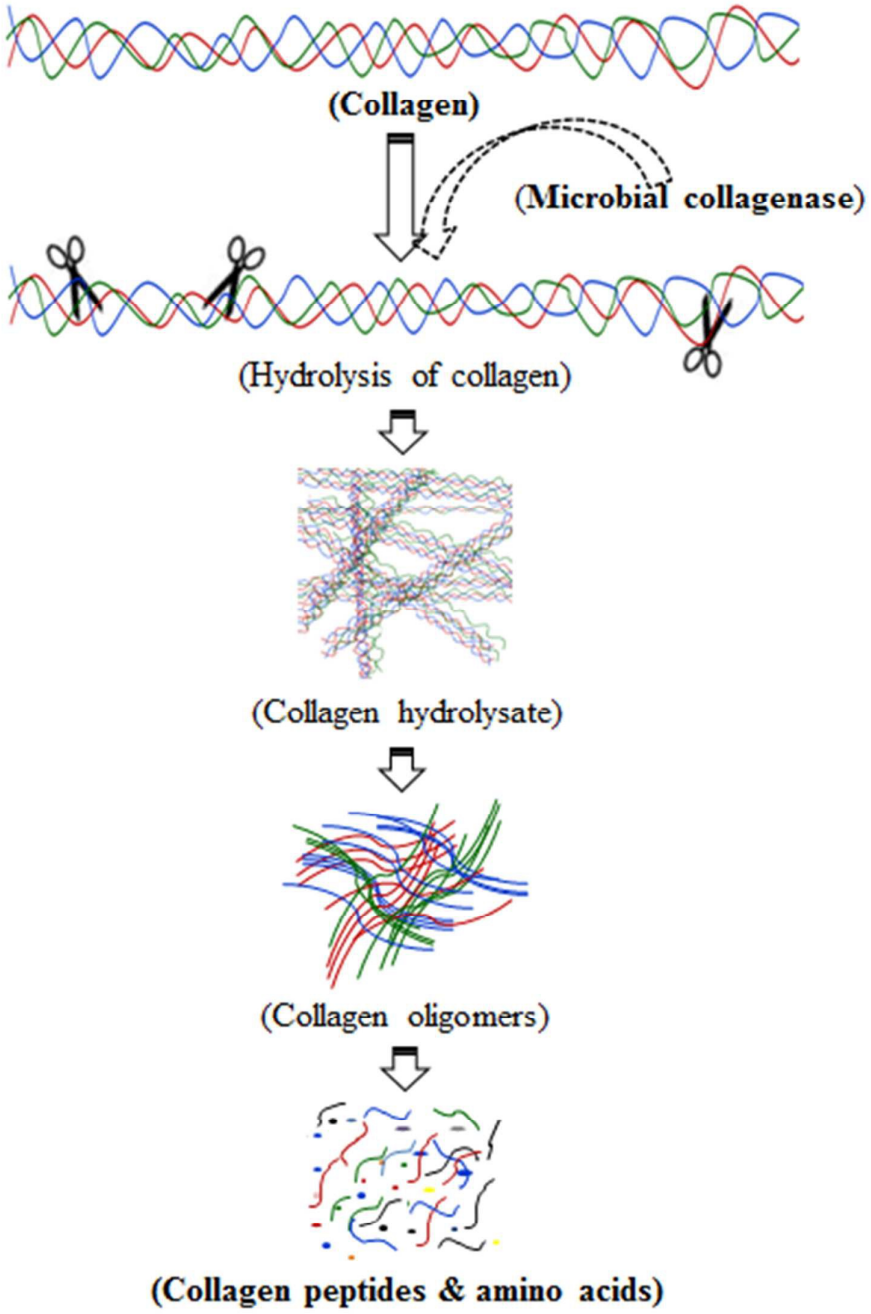


Fig. 4

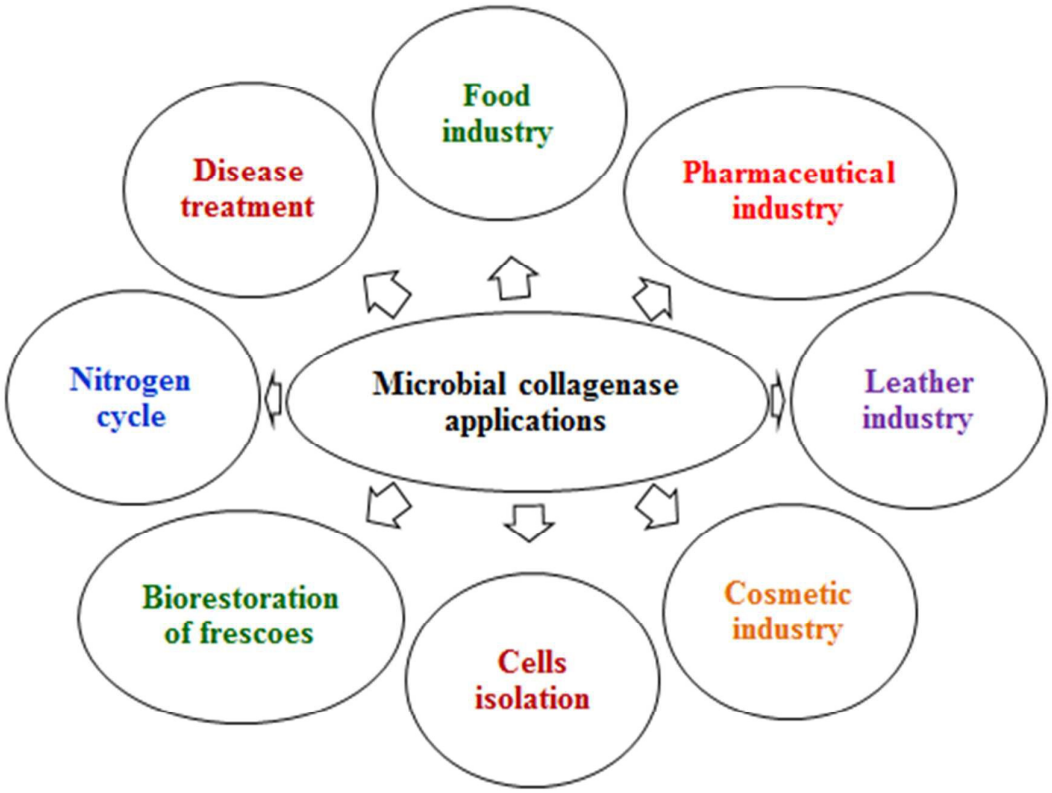
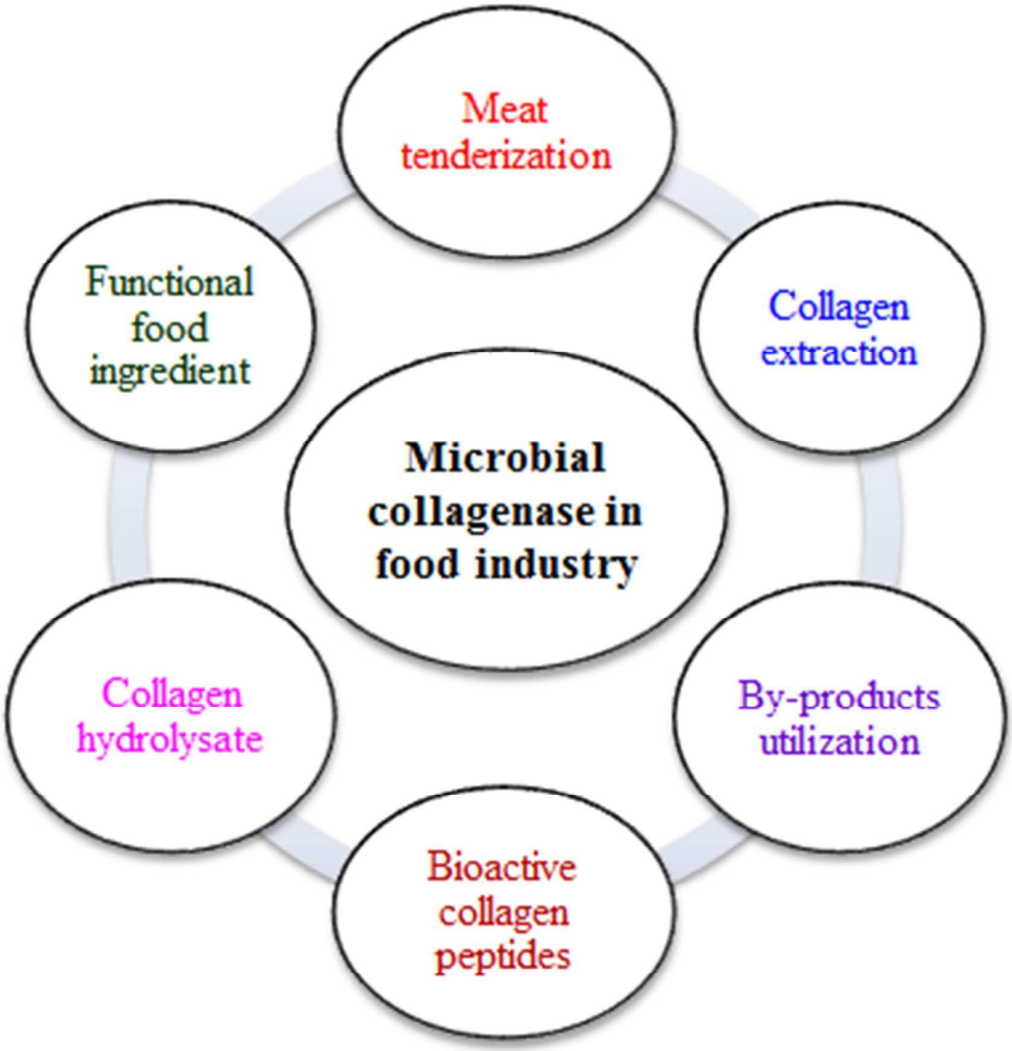


Fig. 5



Graphical abstract