



Industrial sustainability of microbial keratinases: production and potential applications

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Abstract

Keratinases are proteolytic enzymes with a particular ability to cleave peptide bonds in keratin, and in other proteins. Due to their broad-spectrum of activity, keratinases are considered viable substitutes for chemical and thermal treatments of protein-rich industrial by-products. Among these protein residues, special attention has been given to keratinous materials (feathers, hair, horns, etc.), which disposal through harsh conditions methods, such as acid/alkaline hydrolysis or incineration, is not considered ecologically safe. Microbial keratinolytic enzymes allow for keratin degradation under mild conditions, resulting in keratin hydrolysates containing undamaged amino acids and peptides. In this review article, we offer perspectives on the relevance of these unique biocatalysts and their revolutionary ascent in industries that generate keratin-rich wastes. Additionally, we share insights for applications of keratinases and protein hydrolysates in agriculture, animal feed, cosmetics, pharmaceuticals, detergent additives, leather processing, and others. Due to the scientific importance of keratinases and their potential use in green technologies, searching for bacterial and fungal species that efficiently produce these enzymes may contribute to the sustainability of industries.

Keywords Feather · Keratynolysis · Keratinolytic enzyme · Peptidase · Protease

Introduction

The global growth of meat consumption over the years has contributed to a considerable increase of meat industry waste, including viscera, skins, meat trimmings, bones, blood and epidermal attachments (feathers, hair, horns, teeth, nails and claws) (Meruane and Rojas 2012). The poultry industries are particularly problematic due to the amount of keratinous waste they create. Continuous accumulation of feathers has resulted in a global problem (Srivastava et al. 2020), that needs to be addressed.

The specific recalcitrance of keratin is a factor that makes difficult its management and recycling. Conventional methods, such as incineration, and alkaline or acid hydrolysis, used for disposal of this biomass, are high cost and not considered ecologically safe. This leads the meat industry to seek for alternative sustainable technologies to treat these residues (Callegaro et al. 2019). From a biotechnological perspective there is potential to transform them into added-value products, such as biofertilizers. Thus, microbial keratinases have emerged as an alternative to the treatment of keratin-rich wastes.

This review highlights biotechnological approaches for the use of microbial keratinolytic enzymes as a way to contribute to the sustainable treatment of keratinous wastes. Research works needed to expand the range of promising applications, considering the environmental and economic importance of this subject are discussed.

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Keratin structure and its production in the world

Keratins are among the most important structural proteins found in vertebrates, constituting the intermediate cytoskeletal filaments of eukaryotic cells and the epidermis and epidermal appendages (*e.g.* feathers, horns, claws, nails, wool and hair) (McLellan et al. 2018; Qiu et al. 2020). Due to the amount of waste generated annually, particular attention has been given to feather keratin, with several studies reporting attempts to recycle and reuse this residue for different applications.

Feathers are the most common by-product of the poultry industry (Tesfaye et al. 2017), with keratin accounting for about 90% of their weight (Latschaw et al. 1994). They can constitute about 5–10% (w/w) of the total chicken weight, and can provide more than 100 g of feathers per broiler chicken (Tesfaye et al. 2018; Jagadeesan et al. 2020). The global production of poultry meat was expected to exceed 100 million tonnes in 2020 (Food and Agriculture Organization of the United Nations (FAO); United States Department of Agriculture (USDA)), with highlights to USA, China and Brazil, which are the global leading producers in this sector (Callegaro et al. 2019). In this scenario, a large amount of feathers keratin can be expected annually.

The specific recalcitrance of keratin is a factor that makes difficult its management. Keratins are proteins that can be classified into different groups according to their physicochemical properties, molecular structure and the resulting epithelial cells. Considering the secondary structure, they can be classified into α -keratin and β -keratin. While α -keratin is the main component of wool, hair, hooves, nails, horns and stratum corneum, β -keratin is present in feathers, claws and beaks of birds, reptilian claws and scales (Feroz et al. 2020).

The high cysteine content in the primary sequence of keratins differentiates them from other fibrous proteins, such as collagen and elastin. Disulfide bonds between cysteine residues occurring within and between keratin polypeptides, and hydrogen bonds and hydrophobic interactions, contribute to their compact conformation and high molecular stability (Wang et al. 2016; Sinkiewicz et al. 2018; Qiu et al. 2020). Therefore, biodegradation by common proteolytic enzymes, such as pepsin, trypsin and papain is hampered, being keratinases the only class of enzymes capable of degrading keratins.

Keratin degradation

The accumulation of keratin-rich waste in landfills has led to environmental concerns. The uncontrolled anaerobic degradation of these materials tends to release ammonia and hydrogen sulfide (Callegaro et al. 2019), which create a threat to nature and human health.

Commonly keratin residues are improperly disposed, through harsh methods such as incineration, acid and alkaline hydrolysis, to degrade keratinous residues, or submitted to high pressure or temperature treatments and used in the production of low quality animal feed (Tesfaye et al. 2017; Sharma and Devi 2018). However, in addition to high energy consumption (thermal treatments), chemical treatments (*e.g.* acid hydrolysis) can damage some amino acids, such as tryptophan (Sinkiewicz et al. 2017; Rajabinejad et al. 2018), and create additional pollution problems by using acid and alkaline solutions (Sharma and Devi 2018).

With the research studies in microbial keratinases increasing in terms of identification, production, characterization and application (Su et al. 2020a), these enzymes represent an alternative to the use of harsh methods in the treatment of keratin residues. The enzymatic degradation of these proteins proved to have an environmentally sustainable contribution to a positive economic development (Nnolim and Nwodo 2021). This can be achieved through microbial cultivation in keratin residues or direct hydrolysis using cell free enzymes. Both approaches dispense with the use of aggressive chemical agents (acids and alkalis) and high energy consumption (Holkar et al. 2018; Silva, 2018a).

Keratinases belong to the peptidases group (Călin et al. 2017)—hydrolytic enzymes that cleave peptide bonds in proteins and peptides (Silva et al. 2017)—being responsible for degradation of keratin, and commonly other proteins. Most of the reported keratinases are alkaline serine or metallo enzymes, with a few reports describing aspartyl keratinases from yeasts (Negi et al. 1984; Lin et al. 1993). In general, the maximum keratinolytic activity range is between pH 7 and 12.5, and up to 80 °C (Qiu et al. 2020).

To achieve high levels of enzymatic degradation of keratin, keratinases, other auxiliary enzymes (other peptidases and disulfide reductases) and/or reducing agents, such as sulfite ions (sulfite, bisulfite and disulfide), are required. Since disulfide bonds are essential to keratin folding, breaking these bonds is a crucial step to facilitate keratinase access to the substrate. The synergy between sulfitolysis and proteolysis (Lange et al. 2016) favor the complete degradation of keratin complex structure (Qiu et al. 2020). In this process, the crystalline and cross-linked structures of keratin are broken resulting in a hydrolysate mainly composed of soluble oligopeptides and amino acids (Holkar et al. 2018).

Keratinase production

Keratinolytic microorganisms have the ability to use keratins as sources of carbon, nitrogen, sulfur and energy for their growth (Callegaro et al. 2019). Prospecting for new keratinases, many studies have used keratinous residues as substrate in fermentative processes, which offer the

double benefit of, (i) low-cost production of extracellular keratinases, which can be tested for different applications, such as leather treatment, detergent formulations, among others; and (ii) economically viable biomass degradation products, such as biofertilizers and animal feed additives from keratin hydrolysates (Nnolim et al. 2020).

So far, challenges in the enzyme yield of scaled-up conventional fermentation processes have led to studies focusing on ways to improve enzyme production (Fang et al. 2019). The heterologous expression of keratinase genes, either by extraction from microbial cell or metagenomic approaches, is an alternative frequently used. Metagenome techniques allow the construction of a gene library directly from environmental samples. This allows to bypass the barrier of microbial cultivation to extract the gene of interest and open opportunities to explore new keratinases (Su et al. 2020a).

To overexpress keratinase and reduce the production time and cost, different strains, including *Escherichia coli* (Jaouadi et al. 2015; Fang et al. 2019; Zhang et al. 2019; Elhouli et al. 2021), *Bacillus subtilis* (Cao et al. 2019; Tian et al. 2019; Gong et al. 2020), and the yeast *Pichia pastoris* (Li et al. 2007), have been used as heterologous expression systems (Elhouli et al. 2021). Many keratinase genes, especially from *Bacillus* species, which are the most studied keratinase producers, were successfully expressed. Examples of native and recombinant keratinases and their application tests, as well as some different keratin-decomposing microorganisms are presented in the Tables 1 and 2.

In addition, recent developments in enzyme technology have shown that several properties of enzymes can be targeted simultaneously through various genetic engineering approaches. Methods, such as directed evolution, DNA shuffling, site-directed mutagenesis, saturation mutagenesis, fusion and truncation, have been used to improve enzyme stability and catalytic performance (Sharma et al. 2019).

Considering the advances in enzyme technology, in the past two decades, an increase in reports mentioning keratinases have been observed. According to the Web of Science Core Collection, from the reports in the topic “keratinases or keratinolytic enzymes” in the timespan 2000–2020, more than 70% of the studies were reported in the last ten years (2010–2020). This shows the rising interest in the topic and in technologies that contribute to the development of knowledge regarding these enzymes.

Following these studies, numerous patents and commercial enzymes used to degrade keratin, such as Versazyme® and Valkerase® (BioResource International, Inc), CIBENZA® DP100 (Novus International, Inc), NATE-0853 and FEED-0001 (Creative Enzymes®), PURE100 KERATINASE (PROTEOS Biotech), Esperase® and Savinase® (Novozymes A/S), among others, have been described (Lange et al. 2016; Nnolim and Nwodo 2021).

Biotechnological applications

In addition to the use of keratinolytic enzymes to solve the environmental problem of keratin disposal, many other possible applications have been described for these enzymes (Table 1) and the protein hydrolysates resulting from their hydrolytic processes (Table 2).

Keratinases for leather treatment and as a detergent additive

Environmental pollution caused by the large number of contaminants and toxic products in the wastewaters during the traditional leather processing, has become a concern. Keratinases have been recognized as effective enzymes to address this problem, being suitable for the hair removal process and presenting an efficient alternative to the harmful chemicals frequently used (Akram et al. 2020).

In the leather making process, excessive collagen hydrolysis must be avoided to maintain the quality of the leather, including softness, elasticity and fullness (Su et al. 2020a). Therefore, an enzyme defined for use as a depilatory agent must have high hair removal activity but low or no collagenolytic and elastinolytic activity. Several reports have successfully exemplified the application of keratinases to leather processing. In the research by Tian et al. (2019), a keratinase from *Bacillus* sp. LCB12 was expressed in *Bacillus subtilis* SCK6 and tested as an alternative to sulfide in the process of hair removal on goat skin. Moreover, enzymatic and chemical (Na_2S and CaO) dehairing processes for cow leather were compared in a study by Akhter et al. (2020), with the best results in hair removal observed using keratinase from *Bacillus cereus*. These are some examples of research works developed in the application of keratinases for hair removal, yet many additional studies can be found in the literature (Table 1).

There are also reports on the use of keratinases for toxicity reduction of leather industry wastewater (Jaouadi et al. 2015; Kalaikumari et al. 2019). Keratinases and keratinolytic microorganisms can be used for wastewater treatment (Zahara et al. 2020) and to clear obstructions in sewage systems (Brandelli et al. 2009).

In the detergent industry, there is a constant search for new enzymes that improve cleanliness, tissue care and antimicrobial properties. Suitable detergent proteases must meet certain requirements, such as their stain removal efficiency, activity and stability at alkaline pH, and their tolerance to the surfactants, and oxidizing and bleaching agents present in the detergents. Research efforts have been directed towards the discovery and engineering of

Table 1 Summary of some recent studies on keratinases and their origin (production by submerged fermentation (SmF) and recombinant technology or commercial enzyme), biochemical properties, and their potential applications

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|--|--|---|--|----------------------|---|---------------------------|
| <i>Ochrobactrum intermedium</i> NKIS 1 | Production by SmF using chicken feathers | 117 A.U./mL ⁽³⁾ | Metallo-serine keratinase exhibited optimal activity and stability at pH 9 and 40 °C (activity above 80% for 2 h). Keratin azure was used as enzymatic substrate Main metallic ions (5 mM) reported to improve (K ⁺ , Na ⁺ , Ca ²⁺ and Mg ²⁺) and reduce (Zn ²⁺ , Fe ²⁺ , Cu ²⁺ and Mn ²⁺) the keratinolytic activity | Enzyme crude extract | Management of keratin residues and production of bioactive peptides | Sharma and Kango (2021) |
| <i>Streptomyces minutiscle-roticus</i> DNA38 | Production by SmF using chicken feathers | 435.8 A.U. ⁽¹⁾ | Keratinase exhibited optimal activity at pH 9 and 50 °C. Keratin was used as enzymatic substrate | 29 kDa | Management of keratin residues | Allure et al. (2015) |
| <i>Arthrobacter</i> sp. NFH5 | Production by SmF using chicken feathers | 26.57 A.U./mL ⁽²⁾ | Keratinase exhibited optimal activity at pH 8 and 40 °C. Soluble keratin (prepared from white chicken feathers) was used as enzymatic substrate | Enzyme crude extract | Management of keratin residues | Barman et al. (2017) |
| <i>Brevibacterium luteolum</i> (MTCC 5982) | Production by SmF using goat hair | 103 A.U./mL ⁽³⁾ | Keratinase exhibited optimal activity at pH 10 and 30 °C. Keratin azure was used as enzymatic substrate | Enzyme crude extract | Management of animal hair waste from the leather industry | Thankaswamy et al. (2018) |
| <i>Fusarium oxysporum</i> <i>Aspergillus</i> sp. | Production by SmF using swine hair Production by SmF using chicken feathers | 243.25 A.U./mL ⁽³⁾ 113.5 A.U./mL ⁽³⁾ | Keratin azure was used as enzymatic substrate | Enzyme crude extract | Management of keratin residues | Preczeski et al. (2020) |

Table 1 (continued)

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|-----------------------------|---|-------------------------------|--|----------------------|------------------------|------------------------|
| <i>Cortolopsis byrsina</i> | Production by SmF using chicken feathers | – | Two groups of keratinases reported: (1) keratinase exhibited optimal activity at pH 7.0–7.5 and 40–55 °C. Keratinase stability up to 50 °C for 1 h (activity above 63%), and pH 10–11 at 4°C for 48 h (activity above 80%); (2) keratinase exhibited optimal activity at pH 9.0 and 55 °C. Keratinase stability up to 50 °C for 1 h (activity above 63%), and pH range 5–11 at 4 °C for 48 h (activity above 75%). Keratin azure was used as enzymatic substrate | Crude enzyme extract | Detergent additive | Duffeck et al. (2020b) |
| <i>Citrobacter diversus</i> | Production by SmF using chicken feathers | – | Metallo-keratinase exhibited optimal activity at pH 8.5–9.5 and 50 °C. Enzyme stability up to 50 °C for 1 h (activity above 50%), and pH range 5–8 at 4°C for 48 h (activity above 75%). Keratin azure was used as enzymatic substrate | Crude enzyme extract | Detergent additive | Duffeck et al. (2020a) |

Table 1 (continued)

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|--------------------------------------|---|---------------------------------|---|----------------------|------------------------|----------------------|
| <i>Arthro bacter</i> sp. KFS-1 | Production by SmF using chicken feathers | 1,559.09 A.U./mL ⁽³⁾ | Metallo-keratinase exhibited optimal activity at pH 8.0 and 60 °C. Enzyme stability up to 60 °C for 2 h (activity above 90%), and pH range 6–9 for 4 h (activity above 95%). Keratin azure was used as enzymatic substrate | Crude enzyme extract | Detergent additive | Nholim et al. (2020) |
| <i>Streptomyces aureofaciens</i> K13 | Production by SmF using wool powder | – | Main metallic ions (5 mM) reported to improve (Mn ²⁺ , Cu ²⁺ and Sr ²⁺) and reduce (Sn ²⁺ , Fe ³⁺ , La ³⁺ , Zn ²⁺ , Pb ²⁺ and Ag ⁺) the keratinolytic activity | 46 kDa | Detergent additive | Gong et al. (2015) |

Table 1 (continued)

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|---|---|---|---|----------------------|--|---------------------------|
| <i>Bacillus aerius</i> NSMK2 | Production by SmF using chicken feathers | – | Serine keratinase exhibited optimal activity at pH 8 and 45 °C. Enzyme stability up to 65 °C for 30 min., and pH range 4–11 for 2 h (activity above 80%). Crushed feathers were used as enzymatic substrate Main metallic ions (5 mM) reported to improve (Ca ²⁺ , Mn ²⁺ , K ⁺ and Na ⁺) and reduce (Hg ²⁺ and Ba ²⁺) the keratinolytic activity | 9 kDa | Enzymatic hair removal (leather dehairing) Detergent additive | Bhari et al. (2019) |
| Keratinase gene from <i>Bacillus</i> sp. LCB12 expressed in <i>Bacillus subtilis</i> SCK6 | Recombinant keratinase | 1,332 A.U./mL ⁽¹⁾ | Serine keratinase exhibited optimal activity at pH 10 and 60 °C. Enzyme stability up to 45 °C and pH range 4.5–11 (activity above 70% for 1 h). Keratin from wool was used as enzymatic substrate Main metallic ions (5 mM) reported to improve (Cu ²⁺ and Co ²⁺) and reduce (Fe ³⁺ and Ca ²⁺) the keratinolytic activity | 30.95 kDa | Enzymatic hair removal (leather dehairing) | Tian et al. (2019) |
| <i>Bacillus cereus</i> | Production by SmF using chicken feathers | Flask culture: 60 A.U./mL ⁽²⁾ Large-scale keratinase production: 136 A.U./mL ⁽²⁾ | Keratinase exhibited optimal activity at pH 10 and 50 °C. Soluble keratin (prepared from chicken feathers) was used as enzymatic substrate | Crude enzyme extract | Enzymatic hair removal (leather dehairing) | Akhter et al. (2020) |
| <i>Bacillus paralicheniformis</i> MKU3 | Production by SmF using chicken feathers | 1,872.5 A.U./mL ⁽³⁾ | Keratin azure was used as enzymatic substrate | Crude enzyme extract | Enzymatic hair removal (leather dehairing) | Kalaikumari et al. (2019) |

Table 1 (continued)

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|--|--|-------------------------------|---|----------------|--|---------------------------|
| Keratinase gene from <i>Actinomyces viridilutea</i> DZ50 expressed in <i>Escherichia coli</i> BL21(DE3)pLysS | Recombinant and native keratinase | 18,800 A.U./mL ⁽⁴⁾ | Serine keratinase exhibited optimal activity at pH 11 and 80 °C. Enzyme stability up to 100 °C and pH range 7–12 (activity above 80% for 2 h). Keratin azure was used as enzymatic substrate Main metallic ions (2 mM) reported to improve (Ca ²⁺ , Mg ²⁺ and Fe ²⁺) and reduce (Zn ²⁺ , Ni ²⁺ , Cd ²⁺ and Hg ²⁺) the keratinolytic activity. The enzymatic activity was completely inhibited by heavy metals: Ni ²⁺ , Cd ²⁺ and Hg ²⁺ | 20 kDa | Enzymatic hair removal (leather dehairing) | Elhouli et al. (2021) |
| Keratinase gene from <i>Bacillus licheniformis</i> expressed in <i>E. coli</i> BL21 | Commercial enzyme (Lyophilised powder K-4519, Sigma Aldrich) | – | Keratinase exhibited optimal activity at pH 11 and 70 °C (free keratinase), and pH 11 and 75 °C (immobilized keratinase). Enzyme stability up to 70 °C for 1 h (activity above 80%), and pH range 8–12 (activity above 65%). Keratin azure was used as enzymatic substrate | 39 kDa | Improvement of wool fabrics | Srivastava et al. (2020) |
| Keratinase gene from metagenome library. Expression in <i>Bacillus subtilis</i> WB600 | Recombinant keratinase | – | – | – | Enzymatic extraction of keratin for production of keratin hydrogel for wound healing application | Su et al. (2020b) |
| Peptidase gene from <i>Nocardia prostrata</i> expressed in <i>Bacillus licheniformis</i> | Commercial enzyme with keratinolytic activity (Ronozyme ProAct – DSM Nutritional Products) | – | Serine peptidase | – | Dag removal from cattle hides | Navone and Speight (2019) |

Table 1 (continued)

| Microorganism | Keratinase origin and keratinous substrate used | Maximum keratinase production | Functional biochemical properties | Molecular mass | Potential applications | References |
|---------------------------------------|---|-------------------------------|-----------------------------------|------------------------------------|--|---|
| <i>Bacillus licheniformis</i> | Commercial enzyme (Cibenza IND900, Novus International, Inc) | - | - | - | Improved extraction of steroids from chicken feathers and keratinous tissue | Alba et al. (2019) Blecher et al. (2021) |
| <i>Bacillus licheniformis</i> | Commercial enzyme (from Prozyn Bio Solutions for life, Brazil) | - | - | - | Combined use of sugarcane fibre and keratinolytic enzymes helps in preventing excretion of hair balls in cats' feces | Miltenburg et al. (2019) |
| <i>Amycolatopsis</i> sp. MBRL 40 | Production by SmF using chicken feathers | - | - | 20.1 kDa (Ker2) 50.1 kDa (Ker1) | Degradation of β -amyloid fibrils | Ningthoujam et al. (2019) |
| <i>Meiothermus taiwanensis</i> WR-220 | Commercial keratinase (Acquired from Sukehan Bioengineering Co., Ltd, Weifang, China) | - | - | - | Enzymatic decolorization of melanoidins from molasses wastewater | Zhang et al. (2019) |

A.U. (Activity Unit): One unit of keratinase activity under the standard assay conditions. Units of keratinase activity defined as the amount of enzyme required to (1) release 1 $\mu\text{g Tyr min}^{-1}$ (Folin-Ciocalteu method measured at 660 nm), or cause increases of: (2) 0.01 absorbance at 280 nm in 1 min.; (3) 0.01 absorbance at 595 nm in 1 h; and (4) 0.01 absorbance at 440 nm in 1 min

proteolytic enzymes that comply with these biochemical properties (Paul et al. 2016).

Since keratinases generally have broad specificity for soluble and insoluble proteins and are active alkaline enzymes, they are attractive detergent agents. This explains why in the past year, the presence of these enzymes in commercial detergents have been tested and reported by several researchers (Akram et al. 2020; Duffeck et al. 2020a, b; Emon et al. 2020; Nnolim et al. 2020).

Keratinases for cosmetic, pharmaceutical and biomedical applications

In cosmetic applications, keratinase have been tested to develop cream formulations for removal of hair and treatment of skin or hair. It was described that keratinase from *Bacillus subtilis* DP1 exhibited compatibility with the cream formulation, resulting in successful hair removal from rabbit ears (Sanghvi et al. 2016). In another cosmetic assay, keratinase from *Bacillus subtilis* AMR was evaluated for the production of a keratin hydrolysate to be incorporated in a shampoo formulation for hair hydration (Villa et al. 2013). The potential of keratinases for treating acne, corn and for callus removal was also suggested (Vidmar and Vodovnik 2018).

In pharmaceutical proposals, keratinases have been tested for degradation of keratinous material for improvement of drug penetration and possibly for application in the treatment of nail disease (Mohorcic et al. 2007). In experiments by Rai et al. (2020) using silver nanoparticles (AgNPs) immobilized with β -keratinase, it was observed that β -keratinase may enhance the bactericidal activity of AgNPs.

Proteolytic enzymes, especially those with a broad specificity spectrum, have been tested for biofilm removal. The antibiofilm effect and the biofilm dispersal activity have been reported for *Stenotrophomonas maltophilia* Kb2 keratinase against pathogenic bacteria *Staphylococcus aureus* MTCC-96 and *Escherichia coli* MTCC-739 (Bhange et al. 2015).

Keratinases can also be applied for extraction of keratin to be used in the production of biomaterials for biomedical applications. For instance, enzymatic extracted keratin was evaluated for its potential in the production of a hydrogel for wound healing application (Su et al. 2020b). Another proposal suggests the use of keratinases for prion decontamination, which could contribute to avoid incineration of medical equipment and samples (Mitsuiki et al. 2006; Okoroma et al. 2013). Ningthoujam et al. (2019) have also reported the use of microbial keratinase for in vitro degradation of β -amyloid fibrils.

Table 2 Microbial keratinases and protein hydrolysates resulting from their hydrolytic processes: microorganisms, production systems (submerged fermentation (SmF), composting media, and recombinant technology or commercial enzyme), protein source and potential applications

| Microorganism | Protein hydrolysate production system | Protein source | Potential applications | References |
|---|--|---|---|-----------------------------|
| <i>Bacillus</i> sp. | SmF | Chicken feathers | Biofertilizer | Nafady et al. (2018) |
| <i>Amycolatopsis</i> sp. MBRL 40 | SmF | Chicken feathers | Biofertilizer | Tamreihao et al. (2017) |
| <i>Streptomyces</i> sp. | Composting media (30 g feathers inoculated with 1–5 mL of <i>Streptomyces</i> sp.) | Chicken feathers | Biofertilizer | Biswas et al. (2020) |
| <i>Streptomyces</i> sp. SCUT-3 | SmF | Chicken feathers | Nutrition source for animals (tilapia) and plants (rice) | Li et al. (2020) |
| <i>Fervidobacterium islandicum</i> AW-1 | SmF | Chicken feathers | Improvement of recombinant protein production (culture of KerK recombinant <i>B. subtilis</i>) | |
| <i>Fervidobacterium islandicum</i> MT1 | SmF | Chicken feathers | Inhibition of collagenase, elastase, and radical scavenging activities. Feather keratin peptides are potential candidates as skin anti-aging agents | Yeo et al. (2018) |
| <i>Bacillus thuringiensis</i> MT1 | SmF | Donkey hair | Supplementation of culture medium for improvement of vitamin B1, B2 and B12 production by <i>Saccharomyces cerevisiae</i> ATCC 64712 | Hassan et al. (2020) |
| <i>Bacillus licheniformis</i> | SmF | Chicken feathers | Production of bioactive peptides with antioxidant activity | Alahyaribeik et al. (2021) |
| <i>Corioliopsis byrsina</i> | Keratinase cell-free | Collagen gelatin | Production of collagen hydrolysate | Duffeck et al. (2020b) |
| <i>Ochrobactrum intermedium</i> NKIS 1 | SmF | Chicken feathers | Antioxidant and free-radical scavenging activities | Sharma and Kango (2021) |
| <i>Chryseobacterium</i> sp. kr6 | SmF | Chicken feathers | Antioxidant activity | Fontoura et al. (2019) |
| <i>Chryseobacterium</i> sp. kr6 | SmF | Chicken feather | Antioxidant, angiotensin-I converting enzyme- and dipeptidyl peptidase-IV-inhibitory activities | Fontoura et al. (2014) |
| <i>Bacillus licheniformis</i> PWD-1 | Commercial keratinase (Cibenza DP100™, Novus International) | Proteins from animal diet (corn-soybean meal) | Animal feed supplementation (weaned piglets) | Wang et al. (2011) |
| <i>Bacillus licheniformis</i> PWD-1 | Commercial keratinase (Cibenza DP100™, Novus International) | Proteins from animal diet (rice bran, cottonseed meal, rapeseed meal, corn dried grains, peanut meal and corn-soybean meal) | Animal feed supplementation (pigs) | Huang et al. (2018) |
| <i>Bacillus licheniformis</i> PWD-1 | Commercial keratinase (Versazyme, BioResource International) | Proteins from animal diet (corn-soybean basal diet) | Animal feed supplementation (broiler chickens) | Wang et al. (2006) |
| <i>Bacillus licheniformis</i> PWD-1 | Commercial keratinase (Versazyme, BioResource International) | Proteins from animal diet (soybean and cottonseed meals) | Animal feed supplementation (broiler chickens) | Wang et al. (2008) |
| <i>Bacillus licheniformis</i> (LMUB05) | Crude and immobilized enzyme | Feather meal-based diet | Animal feed supplementation (broiler chickens) | Adetunji and Adejumo (2018) |
| <i>Bacillus licheniformis</i> PWD-1 | Commercial keratinase (Versazyme, BioResource International) | Proteins from animal diet (sorghum and corn) | Animal feed supplementation (pigs) | Chen et al. (2017) |

Table 2 (continued)

| Microorganism | Protein hydrolysate production system | Protein source | Potential applications | References |
|--|---------------------------------------|------------------------|------------------------------|------------------------|
| <i>Bacillus amyloliquefaciens</i> 3–2 | Crude enzyme | Feather meal | Animal feed supplementation | Zhou et al. (2020) |
| Recombinant <i>Bacillus megaterium</i> | SmF | Chicken feathers | Biogas production (methane) | Forgaács et al. (2011) |
| <i>Bacillus</i> sp. C4 (2008) | SmF | White chicken feathers | Biogas production (methane) | Patinvoh et al. (2016) |
| <i>Bacillus licheniformis</i> KK1 | SmF | Feather meal | Biogas production (hydrogen) | Bálint et al. (2005) |

Biotechnological applications of protein hydrolysate

Protein hydrolysates are complex mixtures of peptides and amino acids resulting from the hydrolysis of a protein rich substrate, and which can then be used for example, as an additive to animal feed, in biogas production and as biofertilizer (Nafady et al. 2018). A resume of different applications for protein hydrolysates produced by keratinolytic enzymes are presented in Table 2.

Animal feed

Keratinases can be used as additives in animal feed for protein degradation, helping improve the digestibility and contributing to increase animal weight.

The effects of corn–soybean diet supplementation with keratinase at 0.05% (w/w) on weaned piglets, were studied (Wang et al. 2011). The experiments were carried out using Cibenza DP100™ keratinase (Novus International, Shanghai, China) from *Bacillus licheniformis* PWD-1, an enzyme with clear affinity for the hydrolysis of soybean protein. While in keratinase absence only 11.81% of glycine and 24.20% of β -conglycinin were hydrolyzed, in the presence of keratinase those values increased to 94.74% and 88.89%, respectively. The hydrolysates resulting from keratinase addition to the animal feed contributed to improve the intestinal morphology and ecology of the piglets. The improvements observed included reductions of *E. coli* and total aerobic counts, and ammonia nitrogen concentration and branched-chain volatile fatty acid content, in the colon and the crypt depth in jejunum and ileum. Additionally, increases in *Lactobacillus* spp. and total anaerobic counts in the colon, the *n*-butyric acid in the cecum and the villus height to crypt depth ratio in the ileum, were detected. The total tract apparent digestibility of dry matter, energy, crude protein and phosphorus were improved, leading to an increase in weight gain and feed conversion. Also, the incidence of diarrhea, one of the causes of weaned piglets death was positively reduced (Wang et al. 2011).

Huang et al. (2018) also demonstrated with pigs that the supplementation of different diets (rice bran, corn dried grains and cottonseed, rapeseed, peanut and corn-soybean meals) with Cibenza DP100™ keratinase at 0.05% (w/w), affected crude protein and most of the analyzed amino acids to apparent and standardized ileal digestibility (AID and SID, respectively). The highest AID and SID for most amino acids were observed for corn–soybean and peanut meals whereas rice bran and corn dried

grains diets presented the lowest values for most amino acids. Although keratinase supplementation contributed to improve amino acids AID and SID for the six diets studied, only in the corn–soybean meal its supplementation significantly improved the crude protein digestibility (Huang et al. 2018).

There are also reports of Versazyme keratinase (BioResource International), obtained from *Bacillus licheniformis* PWD-1, being used as a feed additive to improve animal digestibility. Chen et al. (2017) reported beneficial effects in protein digestion by pigs due to addition of Versazyme to sorghum and corn-based diets, with observed increases in crude protein AID and villus height to crypt depth ratio in duodenum. For broiler chickens, Versazyme supplementation improved feed conversion ratio, breast meat yield and body weight (Wang et al. 2006). Moreover, feed components (e.g. starch) digestibility and intestinal development of chickens were improved in the presence of the enzyme (Wang et al. 2008).

Recently, an increase of free amino acids and soluble peptides content was observed in the feather meal treated with the compound enzymatic hydrolysis (CEH) from *B. amyloliquefaciens* 3–2. The protein solubility and in vitro digestibility also increased 20.75 and 10.27 times, respectively. These results suggest that CEH can be a promising approach to improve the nutritional value of feather waste (Zhou et al. 2020).

Biogas production

Anaerobic digestion is a promising biological process for renewable energy production from a variety of waste substrates, such as feathers. The anaerobic degradation of feathers traditionally occurs at thermophilic or mesophilic conditions, usually with mixtures of different types of waste (manure, mixed bone fractions and offal). These materials are hydrolyzed to amino acids, which are then converted into organic acids, ammonia, carbon dioxide, hydrogen and minor amounts of sulphur compounds. Among them, acetic acid, carbon dioxide and hydrogen are essential for methane production (by methanogenesis) (Vidmar and Vodovnik 2018).

The biological pre-treatment of feathers with the recombinant *B. megaterium* (carrying keratinase gene from *B. licheniformis*), followed by anaerobic digestion using an inoculum from solid waste digester (Borås Energi and Miljö AB, Sweden), resulted in a methane production of about 0.4 Nm³ CH₄/kg volatile solids (VS) which corresponds to 222% of the methane yield obtained on feathers without biological treatment (0.18 Nm³/kg·VS) (Forgács et al. 2011). Another similar study showed that chicken feathers pre-treated by *Bacillus* sp. C4 (2008), a bacterium capable of producing

α- and β-keratinases, were successfully used as substrate for methane production. In comparison to anaerobic digestion of untreated feathers, an improvement in methane yield was achieved when using feathers hydrolysate as a substrate in anaerobic culture with sludge or granules of bacteria, which resulted in increases of 292% and 105%, respectively (Patinvoh et al. 2016). Beyond methane production, Bálint et al. (2005) provided evidence that hydrogen can also be obtained by biological degradation of keratin-rich waste. The bacterium *Bacillus licheniformis* KK1 was used to convert feathers into a fermentation broth rich in amino acids and peptides, with further metabolization by *Thermococcus litoralis*. The growth of *T. litoralis* in the keratin hydrolysate resulted in the production of biohydrogen, a by-product of the fermentation (Bálint et al. 2005).

Biofertilizer

The global demand for food is a boost for the use of synthetic fertilizers, mainly for the supply of nitrogen (N) in the soil, the main limiting nutrient for plant growth. As alternative, plant and animal biomass, such as feathers, are great sources of nitrogen and could be used as biofertilizers in agriculture to reduce the excessive use of conventional inorganic fertilizers (Silva 2018b).

Although feathers contain almost 15% (w/w) of N, their recalcitrance leads to a slow degradation and mineralization of N in the soil, making it difficult to use them directly as a fertilizer (Jain et al. 2016). However, hydrolysates obtained from enzymatic or microbial processing of feathers are rich in peptides, amino acids, and some minerals (P, K, Ca, Fe, Mn, Zn, Cu) related to feather composition and can be used as natural plant fertilizers (Kshetri et al. 2019; Nurdawati et al. 2017).

Keratin hydrolysates have the benefit of improving soil microbial activity (Rai and Mukherjee 2015). The protein hydrolysates may be mineralized by the soil microbiota, releasing nitrogen that can be absorbed by the plants (Nurdawati et al. 2019). Peptides and amino acids can also be directly absorbed by plant roots and leaves, being translocated to other plant tissues and acting as growth stimulants. Tryptophan, is a fundamental amino acid for the synthesis of indolacetic acid (IAA), a hormone with important plant growth functions (Kshetri et al. 2019).

In a study by Nafady et al. (2018), the bacterium *B. licheniformis* was capable of degrading feathers, from which L-tryptophan was used to produce IAA. Other amino acids including valine, isoleucine, proline, tryptophan, alanine, asparagine, serine and glycine were detected during feather decomposition.

The production of IAA by *Thermoactinomyces* sp. was evaluated by Verma et al. (2016). Even in medium without

L-tryptophan supplementation, it was possible to produce the phytohormone IAA solely from the tryptophan present in the feathers hydrolysate. The feathers hydrolysate was also tested as a soil biofertilizer through evaluation of *Cicer arietinum* seed germination, growth and development. In comparison to the control experiment, earlier germination of seeds and higher plant growth were observed in soil supplemented with feather culture lysate.

In submerged feather cultures, Jeong et al. (2010) also observed that the Gram-negative bacterium *Stenotrophomonas maltophilia* R13 was capable of degrading feathers and produce IAA without L-tryptophan supplementation. It was observed that the keratin hydrolysates stimulated *S. maltophilia* R13 growth, exhibiting a zone of clear inhibition to the growth of phytopathogenic fungi, including *Botrytis cinerea* KACC40574, *Colletotrichum gloeosporioides* KACC40812, *Fusarium oxysporum* KACC40038 and *Pythium ultimum* KACC41062 (Jeong et al. 2010).

Other reports on the use of keratin hydrolysates as biofertilizers are referred in Table 2.

Other potential applications of keratinases and keratin hydrolysates

Recent reports have suggested the use of keratinase for removal of dag from cattle, sheep and goats. Dag are recalcitrant deposits composed of organic matter, including feces, hair, soil, urine, feed and straw (Navone and Speight 2020). Their removal from the animal before slaughter can reduce microbial meat contamination and leather skin damage. The use of keratinase can weaken the interactions of dag with animal hair and contribute to the access of other enzymes (e.g. cellulase, xylanase, lipase, laccase) to the dag, favoring its removal by washing (Navone and Speight 2019).

The improvement in vitamin B1, B2 and B12 production by *Saccharomyces cerevisiae* ATCC 64712, through culture medium supplementation with donkey hair hydrolysate, was reported (Hassan et al. 2020). The hair-keratin hydrolysate was produced using keratinase from *B. thuringiensis* M. strain.

The commercial keratinase Cibenza® IND900 (Novus International, Inc., St. Charles, MO) was also suggested to improve the glucocorticoids extraction from chicken feathers (*Gallus gallus domesticus*) to be used in analysis of biological stress response (Alba et al. 2019). Blecher et al. (2021) also described the use of this keratinase for the extraction of reproductive steroid hormones from Temminck's pangolin (*Smutsia temminckii*) scales.

In the textile industry, keratinases can be used for treatment of wool fabrics, improving their hydrophilicity, dyeing and shrinkage properties (Gunes et al. 2018; Srivastava

et al. 2020). In another different application, keratinase was also tested to decolorize melanoidins from molasses wastewater (Zhang et al. 2019).

Biological activities of feather hydrolysates have also been described. Kshetri et al. (2020) reported antioxidant and antityrosinase activities in hydrolysates produced using a keratinase from a *Bacillus* sp. strain. Compounds presenting antityrosinase and antioxidant properties have wide applicability in the cosmetic, pharmaceutical and food industries. The hydrolysis of chicken feathers by *Chryseobacterium* sp. kp 6 resulted in hydrolysates rich in peptides with antioxidant and antihypertensive activities (Fontoura et al. 2014). Feather keratin hydrolysate have also been described as having the ability to reduce highly toxic forms of chromium, Cr (IV) to their less toxic form, Cr (III) (Bhange et al., 2016), and radical-scavenging activity and ferric reducing power (Łaba et al. 2018).

Future perspectives

Microorganisms have been intensively studied and pointed as valuable sources for production of different enzymes. The exploitation of microbial diversity has allowed to process complex biomass residues, such as lignocellulose, chitin and keratin, and create added-value products. In the sustainable technologies horizon, the bioprospecting of enzymes assumes a prominent position, considering the microbial species still unexplored. In the scope of keratinase, it is important that new prospective studies continue to be developed.

The bioprocessing of keratin wastes can contribute to solve environmental problems related to their disposal, creating opportunities for the application of keratinases and protein hydrolysates in agriculture, animal feed, cosmetics and pharmaceuticals, among others. Although several studies report the successful production, purification and application of keratinases on a laboratory scale, it is still necessary to exploit new strategies to improve production yields in order to satisfy the increasing industrial demands. Therefore, metagenomics, protein engineering and heterologous gene expression studies are essential to expand findings on these enzymes and promote their production at large scale.

Declarations

Conflict of interest The authors declare no financial or commercial conflict of interest.

Ethical approval In this article, we did not perform any studies with human participants or animals.

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