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Microbial Keratinases: Enzymes with Promising Biotechnological Applications

Beti Vidmar and Maša Vodovnik*

Chair of Microbiology and Microbial Biotechnology, Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia

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*Corresponding author:
Phone: +38613203955;
Fax: +38617241005;
E-mail: masa.vodovnik@bf.uni-lj.si

ORCID IDs: 0000-0001-8822-4962 (Vidmar), 0000-0002-1189-2417 (Vodovnik)

SUMMARY

Keratin is a complex and structurally stable protein found in human and animal hard tissues, such as feathers, wool, hair, hoof and nails. Some of these, like feathers and wool, represent one of the main sources of protein-rich waste with significant potential to be transformed into value-added products such as feed, fertilizers or bioenergy. A major limitation impeding valorization of keratinous substrates is their recalcitrant structure and resistance to hydrolysis by common proteases. However, specialized keratinolytic enzymes produced by some microorganisms can efficiently degrade these substrates. Keratinases have already found a purpose in pharmaceutical, textile and leather industries. However, their wider implementation in other processes, such as cost-effective (pre)treatment of poultry waste, still requires optimization of production and performance of the available enzymes. Here we present a comprehensive review covering molecular properties and characteristics of keratinases, their classification, traditional and novel approaches in discovery of novel enzymes, production, characterization, improvement and biotechnological applications.

Key words: keratin, keratinase, waste degradation, keratinolytic microorganisms, biotechnological applications

INTRODUCTION

Corneous (hornlike) tissues have a specific role in vertebrates, representing a protective barrier between the organism and its environment. The major building blocks, defining properties such as the degree of rigidity and hardness of corneous tissues, are specialized structural proteins named keratins (*1*). Due to their protective role, the structure of these proteins is very recalcitrant and resistant to the degradation by widespread enzymes (*2*). While this property is primarily an evolutionary advantage of humans and animals possessing corneous tissues, it also represents a significant challenge for industrial waste management (*3*). Industrial poultry processing (feathers) and sheep farming (low quality wool) produce large amounts of keratin-rich waste. This type of protein-rich waste holds a significant potential for the transformation into value-added products or bioenergy (*4*). However, current mainstream strategies of keratinous waste valorization involve physical/chemical (pre)treatment of the substrates that are not environmentally friendly. A promising alternative approach that might solve this issue would be the application of cost-effective proteolytic enzymes, specialized for the degradation of these substrates (*3*). Although the potential of keratinases for various biotechnological applications has long been recognized, the knowledge on these enzymes and their mechanisms of action on the substrate, production and optimization still needs to be expanded in order to make their industrial use feasible. The aim of the following review is to approach comprehensively current knowledge on various aspects of these enzymes and their production that may be helpful for future research.

KERATIN AS A SUBSTRATE

The three-dimensional structure of the first keratin molecule was first described in 1959, but the number of studies in this area increased extensively after 1990 (*5*). Currently, there are at least 30 different keratin polypeptides classified into various groups, depending on

their unique physicochemical properties, molecular structure and the producing epithelial cells (*6*). Keratins are resistant to degradation by conventional proteases (pepsin, trypsin) and insoluble in diluted acids, alkaline reagents, water and organic solvents (*7*). The amino acid sequence and composition of keratins affect their folding, properties and functions of keratin filaments. Cysteine plays a key role in the formation of disulphide bonds, important for structural stability of these molecules (*6*). Hard keratins (found in hard excrescences, such as feathers, hair, nails and hooves) contain more disulphide bonds than soft, flexible keratins. Amino acid sequence also affects the secondary structure of keratins, which may be enriched in α-helix (typical for α-keratins) or β-sheet structures (typical for β-keratins) (*8*).

Keratins typically consist of three domains with different secondary structures: head domain, central helical domain and tail domain (*6*). Head domain, or N-terminal part of protein, is a globular structure with β-turns consisting of variable number of amino acids (50–100) with a positive net charge. Central helical domain of α-keratins comprises approx. 310 amino acids and consists of four right-handed α-helical subdomains (1A, 1B, 2A and 2B), which are separated from one another by non-helical β-turns called "linker" regions. The latter differ in their amino acid sequence and length (*9*). Approximately 38–45 % keratin molecule is in the α-helical conformation (*10*). Tail domain or C-terminal part of protein contains globular structure. The domains and subdomains of one keratin molecule interact with those of adjacent keratin molecules forming heterodimers, tetramers and, finally, keratin fibres. The rod domains of one acidic and one basic keratin molecule in parallel orientation form heterodimers of keratin molecules, and represent the building blocks of keratin filaments (*11*).

Quaternary structure of keratins has an effect on the capacity for the formation of a tetramer (protofilament with a diameter of 2 nm) (*12*), an octamer (protofibril, two protofilaments, with a diameter of 4.5 nm) (*13*) and unit length filaments (ULFs) with a diameter of 20 nm (*14*), comprising four bundled protofibrils (*15*). Structures of protofibrils and microfibres are strengthened by disulphide bonds, hydrogen and hydrophobic interactions, which also play a crucial role in rigidity and recalcitrance of keratin molecules (*16*).

KERATINASES: PROTEASES WITH SPECIFIC PROPERTIES AND MODE OF ACTION

Proteases are ubiquitous in nature as they are involved in many biological reactions, both at cellular and whole organism levels and have an important ecological role in circulation of nitrogen in nature (*16–18*). Proteases are classified as hydrolytic enzymes that may either cleave peptide bonds near the ends of polypeptide chain (exoproteases) or inside the chains (endoproteases). According to the amino acid sequences in the enzyme active sites and associated catalytic mechanisms, proteases can be classified into seven broad groups: serine, cysteine, threonine, aspartic and glutamic proteases, metalloproteases and asparagine peptide lyases (*17,19*). The sequencing era introduced the need for upgraded classification system based on sequence similarities, also reflecting evolutionary relationships between the proteolytic enzymes. Considering this structure-based classification, the database MEROPS was established, in which peptidases are currently organized into 62 clans and 268 families (MEROPS, edition 12.0, 2018) (*20,21*).

Keratinases (EC 3.4.21) are the only group of proteases with a wide temperature and pH range that allow complete degradation of complex and recalcitrant proteins (*8*). The unique characteristic that distinguishes keratinases from other proteases is the ability to bind to the complex and insoluble substrates (feathers, wool, silk, collagen, elastin, horns, stratum corneum, hair, azokeratin and nails) (*8*). Although the mechanism of enzyme adsorption is not yet well understood, it is known that the higher the adsorption capacity, the higher the degree of keratin hydrolysis (*22*). After enzyme binding and cleavage of disulphide bonds, keratin changes its conformation and exposes multiple sites for hydrolytic action of the enzymes (*21*). According to the nature of their active site, keratinases belong to serine- and metalloproteases or serine metalloproteases (*8*). Serine proteases are functionally rich and diverse group of proteases with nucleophilic serine residues (Ser) located in the enzyme active site. The latter attacks carbonyl part of the peptide bond in order to form an acyl-enzyme intermediate (*23*). More than 333 000 serine proteases are known so far and classified in 53 families and 16 clans (MEROPS, edition 12.0, 2018) (*24*). In metalloproteases, nucleophilic attack on the peptide bond can be transmitted through water molecules coordinated by the enzyme's divalent metallic ion (usually Zn(II) and occasionally cobalt or manganese) or bimetallic centre (two Zn(II) ions or one Zn(II) and Co(II)/Mn(II) ion) (*22*). Depending on the required number of metal ions for catalysis, metalloproteases can be divided in two groups: first group requires two metal ions with co-catalytic mode of action, while the second group only requires one (*22*). Approximately 294 000 metallopeptidases have been identified so far and classified into 73 families and 15 clans (MEROPS, edition 12.0, 2018) (*24*).

Keratinases are robust enzymes with diverse biochemical properties. Most of them are monomeric, although there are descriptions of some multimeric enzymes (*25*). Molecular mass of bacterial keratinases ranges from 18 (enzyme SK1-02 from *Streptomyces albidoflavus*) to 200 kDa (enzymes from *Kocuria rosea* and *Fervidobacterium islandicum*), while the enzymes of pathogenic fungi can reach up to 440 kDa (for example keratinase II of *Trichophyton mentagrophytes*) (*8,24,25*). Higher molecular masses are often characteristic of metalloproteases and originate from thermophilic microorganisms (*21*).

METHODS OF DETECTION OF KERATINOLYTIC ACTIVITY

Qualitative and semi-quantitative methods

Qualitative methods for keratinolytic activity detection on solid matrix are usually appropriate for the initial assessment of the samples either when screening for keratinolytic strains

or enzyme fractions. Commonly known assays for protease activity detection apply agar/agarose plates, with incorporated target substrate (*26*). A commonly applied variation of this method is the radial diffusion test, where the proteolytic activity is proportional to hydrolysis zones around small holes in agar plate containing immobilized substrate (*27*). This type of screening media used different substrates, including *e.g.* 0.1 % wool (*28*), feather meal (*25*), hair (*29,30*), keratin powder, soy flour, *etc*. The applied substrates typically also act as enzyme inducers, however, it is essential that the screening medium be free of repressors and inhibitors of target enzymes such as pepstatin, high concentrations of dextrose or glucose (*31*).

Zymography is used as a semi-quantitative method for keratinase detection on solid phase, since it has several advantages over other methods (*32*). This highly sensitive method allows the identification of specific active proteases in complex enzyme mixtures. While the rest of the qualitative and semi-quantitative methods serve exclusively for screening tests, zymography offers the information on molecular masses of the separate enzymes in the extracts (*33*).

Quantitative methods

After initial isolation of keratinolytic strain or specific enzyme(s), quantification of keratinolytic activity under different conditions is usually of interest as a key part of further target characterization. Quantitative methods allow exact determination of proteolytic activity in a liquid phase (spectrophotometry, fluorimetry, radiometry, chromatography, capillary electrophoresis and enzyme-linked immunosorbent assays are generally used) (*32*). Keratinolytic activity in protein extracts may be determined using a variety of insoluble chromogenic substrates (examples are keratin azure, azocasein, azokeratin, *etc*.) (*34,35*). Enzyme extract is typically mixed with the substrate suspension and incubated at the appropriate temperature. Afterwards, centrifugation of samples removes the insoluble substrate and the absorbance at the appropriate wavelength detects the release of the product (dye) (*32*).

THE MECHANISM OF MICROBIAL KERATIN DEGRADATION

Despite the resistance to most proteolytic enzymes, keratins do not accumulate in nature, which indicates the presence of naturally occurring keratinolytic microorganisms. These constantly contribute to the recycling of the carbon, nitrogen and sulphur by the degradation of vast amounts of keratinous waste (hair and bird's feathers, *etc*.) (*8,36*).

Cleavage of peptide bonds in compact molecules, such as keratins, is challenging, particularly due to insolubility and difficult access of target peptide bonds. Enzymatic degradation of keratin is a multistage process that requires the following steps: (*i*) adsorption of the keratinases to the surface of macromolecule by electrostatic and hydrophobic interactions, followed by (*ii*) catalytic action. Multistage process of keratin degradation includes two major processes: sulfitolysis or reduction of disulfide bonds and proteolysis (*8,37*). Sulfitolysis can only take place in the presence of reducing compounds such as sodium sulfide, dithiothreitol (DTT), mercaptoethanol, glutathione, cysteine, thioglycolic acid or disulfide reductases, which act in cooperation with keratinases in the degradation of keratin molecules (*8,38*).

REGULATION OF MICROBIAL EXPRESSION OF KERATINASES

Microbial production of proteolytic enzymes is a complex and highly regulated process, dependent on the microbial growth stage (*39,40*). Most keratinases are considered inducible enzymes, but some are also expressed constitutively (*41,42*). Nevertheless, it is important to note that constitutive expression of proteases is mostly associated with caseinolytic and not keratinolytic activity, and it is suggested that the keratinolytic enzymes are mainly inducible (*8*).

The production is typically most intensive at the end of the exponential and/or in stationary phase, which is associated with the adaptation to the lack of nutrients (*40*). This suggests that the production or secretion of proteases may be regulated by nutritional stress, such as the lack of carbon and nitrogen sources. The lack of amino acids in the medium typically affects the cellular GTP concentrations. Under the conditions of high nutrient availability, the concentration of GTP is very high, thus maintaining a transcriptional repressor CodY in phosphorylated/active form. The binding of the repressor to the operator prevents transcription of the gene encoding for specific enzyme. Limitation of nutrients reduces GTP concentrations and consequently inactivates CodY (*43*).

MICROBIAL SOURCES OF KERATINASES

Keratinases are ubiquitous in nature and can be found in representatives from the three domains of life: Eukarya, Bacteria and Archaea. The degradation of keratin is more abundant among Gram-positive than the Gram-negative bacterial genera (*8*). The ability of keratin degradation has so far been reported for strains of *Bacillus licheniformis* (*44*), *B. pumilus*, *B. cereus*, *B. subtilis* (*45*) and non-sporogenic bacteria *Stenotrophomonas* sp. (*38*), *Fervidobacterium pennavorans* (*46*), *F. islandicum* (*23*), *Lysobacter* sp. (*35*), *Nesterenkonia* sp. (*47*) and *Kocuria* sp. (*48*). There are also reports that representatives of some Gram-negative genera degrade this substrate: *Vibrio* sp. (*49*), *Xanthomonas* sp. (*50*), *Thermoanaerobacter* sp. (*51*), *Stenotrophomonas* sp. (*52*) and *Chryseobacterium* sp. (*53*). Descriptions of thermophilic and extremophilic keratinolytic representatives from genera *Fervidobacterium* sp. (*54*), *Thermoanaerobacter* sp. (*51*) and *Bacillus* sp. (*55*) are also available.

In addition, at least 300 known fungal species have been reported so far to use keratin as a nutrient source (*56,57*). Keratinolytic fungi represent dermatophytes (*Microsporum*, *Epidermophyton* and *Trichophyton*) and related species of the genus *Chrysosporium*. Non-dermatophytic keratinolytic fungi are found in two genera, *Chrysosporium* and *Myceliophthora*

(anamorph) (*56*). Most keratinolytic groups of fungi belong to fungi imperfecti, including the following genera: *Aspergillus*, *Alternaria*, *Trichurus*, *Curvularia*, *Cladosporium*, *Fusarium*, *Geomyces*, *Gleomastis*, *Monodictys*, *Myrothecium*, *Paecilomyces*, *Stachybotrys*, *Urocladium*, *Scopulariopsis*, *Sepedonium*, *Penicillium* and *Doratomyces* (*8*). The primary steps of keratin degradation by fungi involve mechanical attack on the substrate by their mycelium in addition to sulfitolysis (*21,58,59*).

INDUSTRIALLY IMPORTANT PRODUCERS OF KERATINASES

The main industrial producers of serine proteases are strains belonging to the genus *Bacillus* (*60,61*). This genus has an important role in white biotechnology for several reasons – it is very well studied, majority of species are non-pathogenic, generally recognized as safe (for example *B. cereus*, *B. stearothermophilus*, *B. licheniformis*, *B. subtilis* and so on) and many functional extracellular proteins are secreted directly into the culture medium (*60,62*). Other promising producers are representatives of the genus *Streptomyces* (*S. rectus* var. *proteolyticus*, *S. griseus*, *S. moderatus* NRRL 3150, *Streptomyces* sp. YSA 2130, *S. diastaticus* SS1, *S. corchorusii* ST36, *S. pactum* DSM 40530) and actinomycetes *Nocardiopsis* and *Oerskovia* (*63–65*). Among the most industrially important psychrotrophic bacterial genera are *Pseudoalteromonas*, *Colwellia*, *Flavobacterium* and *Shewanella*. The enzymes from these organisms are effective at low temperatures due to adaptive structural flexibility (*66,67*).

Archaea represent an important source of extremophilic proteases for biotechnological applications as well as fundamental research. Brandelli *et al.* (*21*) have reported a hot spring isolate, belonging to Desulfurococcales (labelled 1507-2), capable of keratin degradation at 70 °C and pH=6.

SEARCH FOR NOVEL KERATINASES VIA ISOLATION OF NOVEL KERATINOLYTIC STRAINS

The industry has a great interest in microorganisms that are capable of producing large amounts of efficient extracellular keratinases (*33,68,69*). Isolation of novel strains producing significant yields of well performing target enzymes is a prerequisite for the development of a viable industrial fermentation process. If the strains themselves are not easy to cultivate, they may represent a source of keratinase genes that can be expressed in another, already established production host. The environment represents an unlimited source of new keratinolytic strains for the implementation of new industrial processes and improvement of the existing ones (*40,70,71*). Usually, peculiar microecosystems in which microorganisms have to adapt to the lack of nitrogen/carbon sources other than keratin represent the best sources of these enzymes (*33,68,69*). During isolation, strains are usually first screened for their keratinolytic potential by qualitative or semi-quantitative methods (screening media), followed by more sophisticated molecular and biochemical methods of characterization (*72*).

Although keratinases from various microorganisms have already been extensively studied, there is still a demand for new ones with improved industrial properties such as higher catalytic efficiency on keratinous substrates, increased stability at elevated temperature and/or pH values and higher tolerance to feedback inhibition (*73*). Apart from strain isolation-dependent methods, other approaches are also in use to increase the number and variability of the available enzymes. These include cloning of genes encoding novel enzymes directly from the metagenomes and modifications of the existent enzymes by protein engineering (**Fig. 1**).

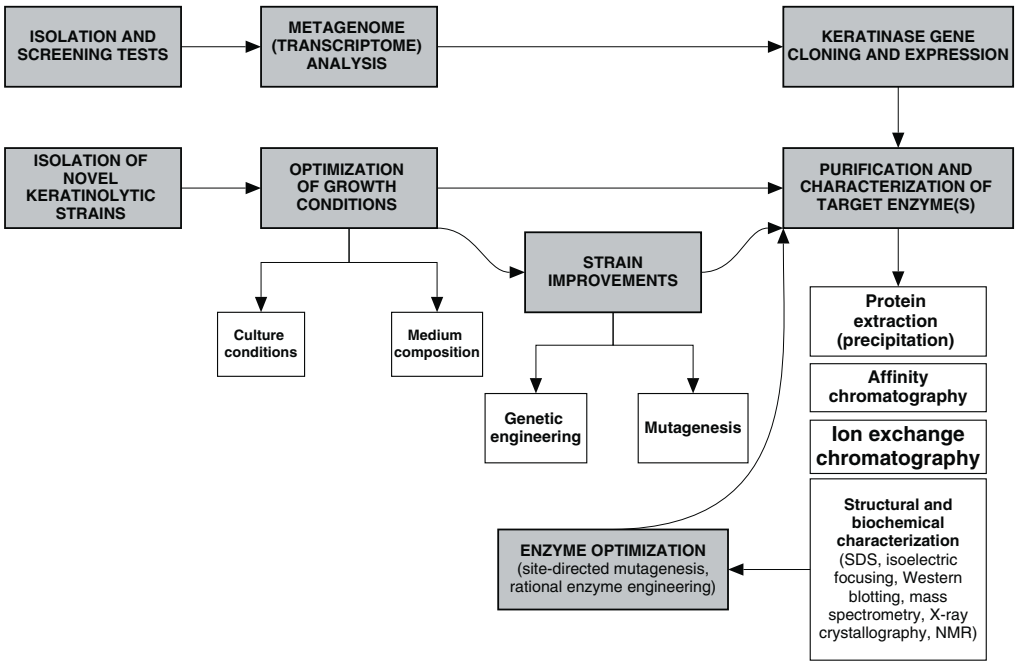

Fig. 1. Different approaches used for identification, production and characterization of novel keratinases and optimization of their performance

CULTIVATION-INDEPENDENT METHODS FOR IDENTIFICATION OF NOVEL KERATINASES

Majority of currently described and applied keratinases originate from isolated microbial strains. However, it is estimated that more than 99 % of microorganisms from natural environments have not yet been isolated, which greatly reduces the number of available microbiological sources (*73,74*).

Sequencing of environmental metagenomes allows us to directly identify genes coding for enzymes with predicted activities *via* comparison to structurally similar sequences in the databases (*75,76*). Moreover, functional metagenomics allows the detection of novel enzymes with no structural similarities to the already known enzymes. In addition, proteomic and transcriptomic tools give us an information on the expression of target genes in specific conditions and explore their mechanisms of action (*77–81*).

Metagenomic approaches

Metagenomics appeared at the end of the last century as an alternative approach to conventional microbial screening of environmental samples (*82,83*). Metagenomic sequence analyses enable the reconstruction of the global enzyme potential in the environmental sample – an information on total consortium of enzymes encoded in examined microbial community (*84,85*).

Research strategies for accessing novel biocatalysts include (optional) pretreatment of genes of interest, nucleic acid extraction, vector and host selection and the screening of metagenomic libraries. Extracting and purification of the sample DNA is a critical step in the process, since the construction of a metagenomic library requires a sufficient amount of high-quality DNA. The next step in building a metagenomic library is the selection of an appropriate vector and host systems, which depends on the characteristics of the environmental samples and intended purpose of the constructed database (*82*).

A selection of proper vector determines whether heterologous DNA will successfully enter the host cells and be actively expressed (the latter is important for functional metagenomics, which is an experimental approach for studying gene function). The choice of a vector system depends on the quality of the extracted DNA and research goals, taking into account the size of inserted fragments, the required number of copies of the vector, type of the used host strain and potential methods of screening. Plasmids, bacterial artificial chromosomes (BACs), cosmids and fosmids are commonly in use (*82*).

Currently, the most widely used cloning and expression host is *Escherichia coli*. However, many eukaryotic genes cannot be functionally expressed in *E. coli*, due to the lack of appropriate mechanisms of post-translational modifications, which makes the development and establishment of new, alternative host systems extremely important (*86*). So far there have been reports of microorganisms such as *Streptomyces* sp. and *Pseudomonas* sp. applied as host strains for the construction of metagenomic libraries (*87*).

An unbiased manner to mine natural microbial communities for new keratinases is by screening expression library for the desired enzymatic activity. To allow for an efficient route for mining new enzymes, high-throughput approaches have to be adapted that will allow to rapidly screen 10^6–10^8 samples (*88*). The enzymatic activity is usually tested on solid matrices supplemented with keratinous substrate. By screening metagenomic library on the plates, positive clones can be identified through a visual examination of discolouration or staining zones around enzyme-producing colonies (*89*). This type of screening does not necessarily require extra devices and can be performed at high-throughput level, but the signals are often poor, which may be the reason for low hit rate. Some alternative approaches can improve the sensitivity of agar-based screening. One strategy with improved sensitivity involves the growth of cell library in microtiter plates, followed by chemical or physical lysis and activity detection (*90–92*). If agar plates are used, targeted activities can be linked to the survival of host organisms, which makes screening or selection sensitive and high-throughput. Another strategy is screening of large libraries by multiple cycles of enrichment using flow cytometry or selective immobilization of active clones (*88*).

The genes encoding target enzymes represent a very small portion of the (meta)genome and in some cases even less than 0.01 % of total sample of nucleic acids derives from environmental sources. Low abundance of target genes in the library plays a key role in the selection of mining strategy (*93*). Metagenomic approaches therefore sometimes involve custom-designed oligonucleotide hybridization probes, which hybridize to specific sequences in metagenomic libraries. For example, catalytic triplet Asp191, His252 and Ser441 are highly conserved regions with a strong preservation in oxyanion region in all keratinases. These regions can be used for designing probes for identification of new keratinases in unexplored sources (*19*).

Another type of target metagenomic enrichment method is stable-isotope probing (SIP) (*94*), in which microorganisms grow in the presence of isotopically labelled substrates. Due to metabolic activity, the isotopes (usually ^{13}C or ^{15}N) are incorporated exclusively in macromolecules of the microbes that metabolize the target substrate (keratin). This increases the target organism's DNA or RNA density that can later be separated from unlabelled molecules (*94*). High density DNA then serves as a template to amplify the functional genes involved in the selected metabolic pathways by PCR, which allows the study of active microorganisms involved in the processes (*95*). Combining pyrosequencing with SIP allows metagenomics of high resolution that targets specific enzymes (*75*).

Transcriptomics

A major disadvantage of approaches based on (meta) genomic library sources of novel enzymes is the high percentage of non-coding and/or non-target DNA regions, which unnecessarily increase the size of the library and (in the case of eukaryotic DNA) need to be removed in order to produce functional

enzymes. In addition to the problems related to the incorrect gene positioning with respect to its promoters, non-excised introns and the differential codons usage, post-translational modifications may also represent a problem when expressing these genes in heterologous hosts (*96*). The advantage of using cDNA libraries is that most of the non-encoding DNA elements are removed, reducing the size of libraries to the genes that are actively expressed in certain conditions (*97*).

MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION OF KERATINASES

Oftentimes, cloning of keratinase genes in a host that can easily be manipulated is essential for ensuring industrial enzyme yields needed for commercialization of the products. Heterologous expression hosts, which are specifically adapted for abundant production of target enzymes, produce more than 50 % of industrially important keratinases. Currently, keratinase expression using heterologous systems would lay the foundation for its genetic manipulation, protein engineering and large scale enzyme preparation (*98*). Properties and original source of the enzyme to be expressed affect the choice of the type of heterologous host (which is usually bacterium or yeast strain). Increased production can be achieved by two strategies: (*i*) inserting the plasmid that contains the gene coding for keratinase under a strong promotor, or (*ii*) using a strain with more keratinase genes integrated in the chromosome.

Despite the fact that *E. coli* is a preferred expression system for recombinant proteins, the bottleneck of producing the protein in this host is accumulation of inactive inclusion bodies and requirement of *in vitro* folding of pro-keratinases, which drastically affects final active enzyme yield (*8,19*). If expression host does not enable correct protein folding, additional procedures have to be introduced for proper formation of disulfide bridges (*99*). Other expression hosts, like *Bacillus* strains, are also used. In these strains, the significant enzyme yield and higher production have been achieved with the introduction and expression of multiple copies of keratinase genes (*e.g.* in *B. subtilis* DB104 and *B. licheniformis* T399D) (*100*). Despite many advantageous features of *Bacillus* sp., the use of this host may be limited due to reported plasmid instability (*100*). In addition, some species (for example *B. licheniformis*) produce significant amounts of indigenous extracellular enzymes (amylases, mannanases, cellulases) which may adversely affect the downstream processes (*101*). Other expression hosts are also being used for heterologous keratinase production, for example yeast *Pichia pastoris*, which provides a good environment for the appropriate post-translational modifications and protein folding of eukaryotic keratinases. In addition, this unicellular eukaryote is simple for culturing and manipulations and has so far been successfully used for expression of many proteases from bacteria, fungi and mammals (*102*). One of the successful examples of heterologous expression of keratinase in *P. pastoris* was the production of *B. licheniformis* MKU keratinase, which exhibited increased thermostability (75 % retained activity at 80 °C) in comparison with the protein produced in *B. megaterium* expression system (*19*). Other examples of heterologous expression of keratinases are in **Table 1** (*38,84,103-107*).

METHODS FOR IMPROVEMENT OF KERATINASE PERFORMANCE AND PRODUCTION

Many times optimization of specific properties of enzymes (such as pH, temperature stability or specificity) or associated genetic elements that regulate their production is necessary to ensure cost-effective production/performance of the target. This can either be done by traditional non-targeted approaches, such as physical (*108*) and chemical mutagenesis (*109*) or by the protein engineering approaches (*110*). Different options are described below.

Physical (UV irradiation-induced) and chemical mutagenesis

Non-ionizing radiation (for example by UV light) results in the excitation of electrons in DNA molecules, which typically results in mutagenic effect. Random mutations in structural gene or regulatory elements may have positive or negative effect on the production and properties of target enzymes. According to Zeng *et al.* (*111*), UV irradiation of *Deinococcus ficus* CC-ZG207 mutants caused a 2-fold increase in keratinase production, in comparison to the wild type strain (*101*).

Table 1. Examples of keratinase genes expressed in heterologous expression systems

Original source of keratinase gene	Accession number of cloned gene	Host strain for cloning and overexpression	Vector type	Reference
Bacillus licheniformis MKU3	DQ071570	*Escherichia coli* BL21	pET30b	(*103*)
Bacillus licheniformis MKU3	DQ071570	*Bacillus megaterium* ATCC 14945	pWH1520	(*103*)
Bacillus licheniformis MZK-05	Q9FDF4	*Escherichia coli* BL21; *Esherichia coli* DH5α	pGEX-6p-2	(*104*)
Bacillus licheniformis PWD-1	S78160	*Bacillus subtilis* DB104	pJCD	(*84*)
Bacillus circulans DZ100	KC621294	*Escherichia coli* BL21	pABT2	(*104*)
Bacillus pumilus KS12	KC814159	*Escherichia coli*	pEZZ18	(*105*)
Geobacillus stearothermophilus AD-11	KJ783444	*Escherichia coli* BL21(DE3)	pTZ57R/T	(*106*)
Pseudomonas aeruginosa	-	*Escherichia coli* AD494(DE3)pLysS	pET-43b(+)	(*107*)
Stenotrophomonas maltophilia BBE11-1	KC763971	*Escherichia coli* BL21 (DE3)	pET22b	(*38*)

Chemical agents, such as ethyl methylsulfonate (EMS), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) or ethidium bromide, can also induce random mutations in DNA molecules. Exposing *B. subtilis* to MNNG, for example, resulted in 2,5-fold higher keratinase activity in mutated strain than in the wild-type (*109*). *Brevibacillus* sp. AS-S10-II treatment with EMS resulted in mutants having a higher growth rate and keratinase production (*101*).

Protein engineering

Molecular methods for improvement of biocatalysts (or regulatory elements affecting their production) is usually performed by one of the following approaches: the rational design or directed evolution.

Rational design

Rational protein design is an effective strategy, which requires the information on the relationship between the sequence, structure and function of target enzymes. It is based on the comparison of the target sequence (predicted structure) to the already characterized enzymes, which allows the prediction and modification of the residues responsible for specific functional properties of the enzyme, substrate and co-factor binding sites, thermostability or enantioselectivity. Novel binding sites for metal ions, active sites, *etc*. can also be introduced into the protein (*106*). Site-directed mutagenesis is a method that applies target changes in double-stranded DNA, resulting in a modified amino acid sequence and tertiary structure of the enzyme (*112*). It is typically used to improve the thermostability, efficiency, activity of keratinases or enzyme yield (promotor mutagenesis) (*103*). Thermostability of *B. licheniformis* BBE11-1 keratinase has been improved by using a computer design (algorithm PoPMuSiC), which was applied for predicting change of free energy of amino acid substitutions. Afterwards, four amino acid substitutions (N122Y, N217S, A193P and N160C) were introduced into the enzyme, using site-directed mutagenesis and mutated genes were later on expressed in *B. subtilis* WB600. The amino acid replacement N122Y caused 5,6-fold increase in the catalytic efficiency, in comparison to wild-type keratinase (*101,104*).

Specific nucleases PstI and KpnI were used for improving stability of serine proteases from *Bacillus* sp., for which oxidation-sensitive methionine 222 was removed and replaced by non-oxidizing amino acid (Ser, Ala or Leu). Consequently, protease mutants, containing modified amino acids, were more resistant to chemical oxidation (*105*).

The relatively rapid PCR-based method used in rational design involves splicing by overhang extension (SOE) (*106*) that generates improved protein variants containing modified (specific) amino acid. This method offers several advantages – it is simple, fast and highly repeatable and does not require restriction enzymes or DNA ligases to generate site-directed mutations (*106*).

Engineering of enzyme immobilization enables the improvement of the efficiency, thermostability and other enzyme characteristics. According to Wang *et al.* (*113*) immobilization of keratinases of genus *Bacillus* sp. can be achieved by fusion of two genes – *kerA,* encoding keratinase, and *stp,* encoding streptavidin, which binds to biotin. Fusion construct *kerA-stp* allowed formation of a recombinant protein keratinase-streptavidin, which was secreted into the medium. Fusion protein was then isolated from the medium using biotinylated beads. This method of immobilization substantially improved the thermostability and pH tolerance of the enzyme (*101*).

Directed evolution

Directed evolution is one of the most effective approaches to tailor biocatalysts for industrial purposes (*106*). Fundamentally, it is an imitation of Darwinian evolution on a laboratory scale, based on sequential random mutagenesis and selection steps (*114*). This approach has already been used to improve a whole series of enzymes for which the structure-function relationship was not known (*115*).

The first step in directed evolution is the introduction of numerous random point mutations by PCR and/or recombination, resulting in the emergence of many molecular variants of a wild-type gene. Point mutations can be introduced using error-prone PCR, for example by substituting Mg^{2+} with other type of cations (*e.g.* Mn^{2+}) or by the application of highly error-prone polymerase. Another possible way of generating novel gene variants is DNA shuffling, which is based on recombination of related genes and also allows the generation of proteins with improved properties. Gene shuffling results in different combinations of hybrid genes, originating from different origin cells or even species (*106*).

Successful selection of engineered enzymes requires a "high-throughput" screening method, which considerably increases the chance of obtaining desired properties and reduces the time and cost of the analysis (*116*). Emerging new technologies that provide sufficient performance are necessary to support the testing of millions of samples in a highly reproducible manner. The most convenient strategy for the detection of enzymes involves the use of chromogenic or fluorogenic substrates (*107*). This strategy is based on the ability of bacteria harbouring the target genes to selectively convert substrates to detectable products. A high-throughput method for single cell screening can be applied by microfluidic droplet sorting yielding improved production hosts for secreted enzymes, which results from an improvement of the cellular functions of the production host. Enzymes producing single cells are encapsulated in 20-pL droplets with a fluorogenic reporter substrate. The platform enables small reagent consumption for a screening experiment, which greatly reduces the costs of screening and engineering of production strains (*107*).

Recent progress in high-throughput enzyme assays has included analytical instruments for parallel screening, thermistor arrays, new fluorogenic and chromogenic substrates, as well as novel applications of pH indicator methods (*117*).

BIOTECHNOLOGICAL APPLICATIONS OF KERATINASES

Microbial enzymes represent a significant share of industrial catalysts, hydrolases taking approx. 65 % of the market (*40*). Among the latter, proteases are an extremely important group of enzymes due to their broad applicability scope (*40,118*). In addition to broad substrate specificity, keratinases stand out from other proteases due to general robustness, which is advantageous in many industrial applications. **Fig. 2** shows different (potential) applications of keratinases, while the most common commercial products already on the market are shown in **Table 2** (*72,119*).

Processing and cleaning of animal hides – leather industry

Leather industry is one of the oldest, fast-growing industries in the world and plays an important role in today's economy. At the same time, it is considered as one of the world's biggest sources of pollution, as leather processing includes use of toxic substances, causing hazardous effects on the environment and workers in industrial plants (*60*).

The treatment of hides involves a series of processes, among which pre-tanning is considered as a major source of pollution (*8*). Chemicals such as sodium sulfide, lime and solid wastes, arising as a result of the preliminary tanning, are the main reason for the increase in biochemical oxygen demand

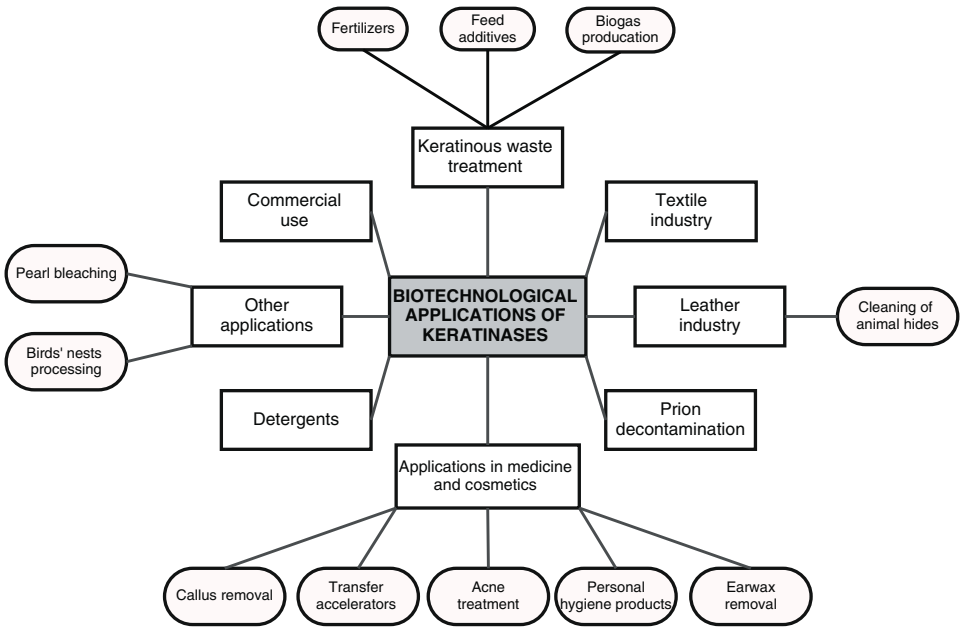

Fig. 2. Biotechnological and industrial applications of keratinases

Table 2. Commercially available keratinase products

Application	Description of the product	Commercial product	Reference
Earwax removal	Successful, safe and effective removal of earwax from the external ear canal	Zymox	(*72*)
Corn and callus removal	Keratinases present natural alternative to the use of acids for corn and callus removal	Keratoclean® Hydra PB, PURE 100 Keratinase	(*72*)
Acne treatment	Acne is caused by blockage of sebaceous glands in the presence of large quantities of keratin, therefore keratinases can be used for successful treatment	Keratoclean® Sensitive PB, Keratopeel® PB	(*72,119*)
Commercial use (poultry feed)	Keratinase product improves the feed ratio and has a positive effect on chicken body mass	Versazyme®	(*72*)
Commercial use (poultry feed)	Enzyme product reduces cost of cooking and temperature processing of feather, therefore increasing the digestibility and nutritional value of feed	Valkerase®	(*72*)
Prion decontamination	Effective decontamination of medical instruments from prions. It contains engineered protease with increased activity, broader specificity and thermostability	Prionzyme™	(*72*)
Biomedical, pharmaceutical and cosmetics	Keratinase product is supposed to regulate the concentration of keratin in pores, therefore helping to eliminate blisters, keratinized skin, it can be used for treatment of dermatophytic and nail diseases, scars and epithelial regeneration	PURE100 Keratinase	(*72*)
Cleaning agents	Cleaning pipes and tanks with different enzymes, including keratinases	Bioguard Plus	(*72*)

(BOD), chemical oxygen demand (COD) and total quantity of dissolved solids (TDS) in wastewater produced in these plants (*8*). The enzyme practice is a green alternative leading to reduction in environmental pollution and improves the leathers (*120*). Proteolytic enzymes are nowadays increasingly used for hide softening to improve pliability of the hides and prepare them for tanning process. Various keratinolytic enzyme preparations for the removal of animal hair without damaging the skin (collagen) are also in use. They selectively degrade the soft keratin tissue in the follicle, thereby pulling out intact hair without affecting tensile strength of the leather (*121*). Enzymes, mainly originating from *Bacillus* sp. (*122–126*), *Pseudomonas stutzeri* (*127*), *Caldicoprobacter algeriensis* (*128*), *Acinetobacter* sp. (*129*), *Paenibacillus woosongensis* (*130*), *Vibrio metschnikovii* (*131*) and different fungi from species *Aspergillus tamarii* (*132*), *Penicillium chrysogenum* and *Trichoderma harzianum* have been reported to be used in leather industry (*133,134*). The application of keratinases in leather industry improves the quality of the final product, and reduces environmental pollution by chemicals, providing a safer working environment (*60,135*).

Detergent and textile industry

Alkaline proteases represent 89 % of proteolytic enzymes used in detergent industry (*8*). Enzymes suitable for use in detergents need to be compatible with other components of the washing agents, and exhibit activity and stability at higher pH values and temperatures. The alkaline keratinase from *Paenibacillus woosongensis* TKB2 is one of the enzymes with potential application in the laundry industry for removing the composite stains without affecting the fabric texture, fibres and strength of clothes (*130*).

Textile industry uses keratinases for processing wool fibres. Wool is composed of structural proteins with high degree of cross-linked disulfide bridges that give fibres mechanical strength and resistance to degradation. This is attributed to the overlapping layers of the epidermis (epi-, exo- and endocuticle) (*136*). Epicuticle is rich in lipids, while the exo- and endocuticle are composed of keratin. The epidermis plays a key role in fibre contraction during washing and dyeing processes. Shrinkage has traditionally been controlled using absorbable organic chlorides. The disadvantages of this process include loss of natural wool character, yellowing of material, pollution of waste water with AOX (adsorbable organically bound halogens), affected biodegradability of fabric (caused by limited bioavailability) and high energy and time consumption (*137*). Environmentally safe alternatives to the chemicals used in this process are enzyme preparations, typically composed of proteases and lipases. The role of proteases is to remove an outer layer of coarse fibre, reducing the rough feeling of the wool. It is necessary to carefully dose the enzymes, as some proteases penetrate deep into fibres, causing their damage, loss of mass and tensile strength (*138*). This is also the reason why currently no process is exclusively based on enzymes. To some extent, penetration can be limited by increasing the molecular mass of proteases aimed for this application by chemical crosslinking, attachment of synthetic polymers, *etc*. (*33*). A better alternative is the use of specific keratinases that selectively act only on keratinous layers of the wool, without adverse effects on the other fibre parts (*137*).

Decontamination of prions with keratinases

Prions are very robust, compact proteins that cause serious neurodegenerative diseases, such as TSEs (transmissible spongiform encephalopathies) (*19*). Due to increased number of prion horizontal transmissions from animals to humans (including iatrogenic transmissions *via* contaminated surgical tools), effective methods for decontamination of material infected with prions are of vital importance. Since conventional methods are based on aggressive and energetically demanding processes, enzymatic degradation of prions may be a promising approach for destruction of these highly aggregated proteins with structural similarities to keratin. So far there have been reports of only two keratinases used for prion decontamination purposes – one isolated from *B. licheniformis* PWD-1 (*137*) and the other from *Streptomyces* sp. (*139*). Nevertheless, in addition to enzymatic treatment, further treatment of infected material with alkaline reagents, detergents and high temperatures is required.

Applications in medicine and pharmacy

Keratinases are also used in cosmetic industry for treatment of acne, calluses, keratinized and dry skin removal, treatment of psoriasis, *etc*. (*140,141*). Applications of keratinases in pharmaceutical industry are mainly associated with improving the passage of fungicidal drugs through the keratinous nail surface. Nail disorders, mainly associated with fungal infections, are ranging from relatively harmless (pigmentation), to painful conditions such as nail dystrophy (*142*). Treatment of fungal infections of nails (onychomycosis) is extremely challenging and traditionally involves long-term consumption of antifungal medicines and repeated monthly injections with corticosteroids, causing many side effects, such as rashes and liver damage (*143*). An alternative form of treatment is a topical application of antimycotic drugs directly to the affected area. The main drawback of this approach is impermeability of the nail surface which affects drug penetration and treatment efficiency. A variety of mechanical (nail abrasions and separation), physical (etching, laser treatment, hydration and fast closing of nails) and chemical methods (keratinolytic agents, such as urea, thioglycolic acid, salicylic acid, N-acetyl cysteine, mercaptoethanol) are being used for improving the medicine transfer to the site of action (*144*). However, these chemicals are only effective in high concentrations and may have a pungent smell (*137*). Keratinases, on the other hand, may be very effective in loosening the nail plates already in low concentrations, which was first demonstrated by keratinase from *Paecilomyces marquandii* (*137,143*).

Transformation of keratinous waste to bioenergy and value-added products

According to regulation (EC) 1774/2002 (*145*) of the European parliament, keratinous waste is classified as the third category of animal products, meaning: (*i*) it is obtained from animal carcases, (*ii*) is not intended for human consumption, and (*iii*) should not transmit diseases to humans or animals (*145*). Large amounts of waste produced by animal processing plants can be used as a substrate for bioenergy and value-added products if properly treated prior to their use. Traditional treatment of keratinous waste includes the use of alkaline and acidic hydrolysis, processing at high pressure and high temperatures (up to 150 °C) (*146*). Despite the aforementioned advantages, these methods are expensive, consume large amounts of energy and lead to loss of some essential amino acids important for subsequent production of value-added products or bioenergy (*33,147*).

Production of feather meal for feed industry, bioenergy production and agriculture

The main waste in poultry industry are feathers, representing 7–10 % of chicken mass. In 2012, around 8.5 billion tonnes of poultry feather were generated worldwide (*148*). Feathers consist of about 90 % keratin (majority being β-keratin) and contain large amounts of serine, glutamic acid, proline and small amounts of methionine, histidine and lysine (*145*). Several million tonnes of feathers per year represent one of the biggest waste byproducts of poultry industry, and an important source of environmental pollution (*8,149*). One of the options for valorization of feather waste involves their transformation to the feather meal, which can be used as a raw material in biodiesel production, an ingredient in bioplastics or as animal feed (*150*). Traditional processing of feathers includes high temperature and pressure, which are the cause of high costs and destruction of some essential amino acids (methionine, lysine and tryptophan). Consequently, final products may be poorly digestible and have a variable nutritional value (*8*). Such shortcomings can be avoided by enzymatic hydrolysis of feathers by keratinases, resulting in production of higher quality amino acids that can be supplemented to poultry, pig, ruminant and fish feeds (*8,150*).

Hydrolyzed feathers can also be converted to bio-hydrogen (*119*), or used as a fertilizer for organic farming that allows slow release of nitrogen, improves plant growth, promotes soil microbial activity, structures the soil and increases its water retention capacity (*8*).

Anaerobic digestion of keratinous waste for the production of biogas

Anaerobic digestion is a promising process for microbial production of renewable energy from different types of waste substrates. During this process, substrates are hydrolysed to amino acids, which are later converted to a variety of organic acids, ammonia, carbon dioxide, hydrogen and minor amounts of sulphur compounds. These are the substrates for methanogenesis – a microbial process in which acetate, hydrogen and carbon dioxide are used to produce methane and carbon dioxide (*151*). Anaerobic degradation of feathers runs under thermophilic (*151*) or mesophilic (*151,152*) conditions and usually includes different types of waste (manure, mixed bone fractions, leftovers and offal).

Theoretical methane yield from proteins is 0.496 m^3/kg of volatile solids (VS) (*153,154*), but only around 0.21 m^3/kg was reported to have been produced from waste feathers (*155*). Low yield is the result of low accessibility of nutrients due to compact keratin ultrastructure in feathers and can be increased by pretreatment (*156*). In recent years different chemical, physical and enzymatic methods of pretreatment are being explored (*155,157*). Development of some recombinant keratinolytic strains with increased production of enzymes aims to increase and improve keratinous waste decomposition before/during anaerobic digestion (*155*). A recombinant *B. megaterium* strain carrying *B. licheniformis* keratinase gene was developed in order to increase hydrolysis of chicken feathers. The recombinant strain effectively degraded feathers prior to biogas production, which resulted in production of up to 80 % of the theoretical methane yield originating from protein digestion. The positive effect is due to the fact that a strong inducible promotor controls the keratinase production in *B. megaterium*, resulting in a prompt and efficient turnout of enzymes without lag phase (*158*).

Other applications

Keratinases are also used in a variety of other applications, including for bioaugmentation of composting of keratin-rich waste (*159*), for modifications of basic structure of fibres in wool or silk (*8*), in different cosmetic products and processing of edible bird's nests. One of the unconventional applications is in pearl bleaching. At the time of bead formation, organic impurities such as free cells, mucus cells and necrotic tissue may be present in the layer of mounting. Therefore it is necessary to improve the quality of beads prior to sale. The pearls are traditionally treated by gentle bleaches (hydrogen peroxide) that can provide soft lightening, although they can offset their colouring and may affect colour irregularity. Zhang *et al.* (*160*) reported using keratinases as an alternative for the traditional-type bleaching process.

CONCLUSIONS

The application of keratinases in some areas of industry is already well established, while they remain a promising tool for agriculture and food industry waste management. The main bottlenecks limiting wider application of many currently available enzymes are their efficiency on complex natural substrates (such as feather and poor-quality wool), low stability and/or high production costs. Different approaches are increasingly emerging to overcome these obstacles with

the goal to make the use of these enzymes for the aforementioned applications cost-effective. Protein engineering and directed evolution are among the most promising strategies to optimize currently available enzymes, while the decrease in the costs of the DNA sequencing is making it possible to expand the consortium of the currently available enzymes with new ones. The development of novel molecular tools may on the other hand enable efficient genome editing of the available and novel producing strains, making the keratinase production more affordable. Future efforts to replace current environmentally questionable practices with enzyme-based treatments of (keratinous) wastes are of significant importance as they may contribute to long-term welfare of humanity, climate change and maybe even survival of the species.

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