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KERATINOLYTIC ENZYMES: PRODUCERS, PHYSICAL AND CHEMICAL PROPERTIES. APPLICATION FOR BIOTECHNOLOGY

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The aim of the review was to analyze the current ideas on keratinases, a group of proteolytic enzymes that catalyse the cleavage of keratins, which are highly stable fibrous proteins. Representatives of various taxonomic groups of microorganisms, including fungi, actinomycetes and bacteria, are keratinase producers. Modern classification of keratinases according to the MEROPS database is given. It based on the similarity of the amino acid sequences, which also reflects the evolutionary interactions between proteolytic enzymes. The MEROPS database combines the proteases into 62 clans and 264 families now. The studies of physical and chemical properties of keratinases indicate that the enzymes are active in a wide range of temperature and pH values, with the optimal action at neutral and alkaline pH and t = 40–70 °C. It was shown that microbial keratinases were predominantly the metallo-, serine- or metallo-serine proteases. They are usually extracellular, and their synthesis is induced by keratin substrates. The review discusses the practical use of keratinases. These enzymes have been successfully applied in bioconversion of keratin wastes to animal feed and nitrogenous fertilizer, as well as in leather, textile, detergent, cosmetic, pharmaceutical industries. Keratinases are also applicable as pesticides and in the production of nanoparticles, biofuel, biodegradable films, glues and foils. In addition, keratinases are used in the degradation of prion proteins which are able to cause a number of human and animal neurodegenerative diseases of spongiform encephalopathy.

Key words: keratinases, producers, regulation of synthesis, physical and chemical properties.

The keratins are unsoluble fibrillar proteins that make up the external protective surfaces in vertebrates and are the structural components of wool, hoof, horns, hair, nails and feathers [1, 2]. Keratins are known for their complex degradation and high stability caused by the firm stabilization of their polypeptide chains, tightly packed with hydrogen bonds and hydrophobic interactions [3]. Moreover, the disulfide bonds cross-linking the chains contribute to their stability and tolerance to the degrading effect of the usual proteases such as pepsin, trypsin and papain [4, 5].

The term “keratin” (derived from Greek “kera”, meaning “horn”) was for the first time used in 1850 to describe matter from the hard, firm tissues forming animal horns and hooves [6].

According to the secondary structure, keratins are grouped in two types:

1) α-keratins contain residues of all amino acids and differ from other fibrillar proteins by their high content of cysteine. Their polypeptide chains are α-helices. The primary structure of the α-keratin polypeptide chains is not periodic. The α-keratins are insoluble and elastic due to the presence of numerous disulfide bonds between polypeptide chains. The protein molecular weight is 10–50 kDa. The main structural element of the mammalian α-keratin is a protofibril with a diameter of 2 nm, formed by three interwoven supercoiled

α-spiral segments of polypeptides with not-coiled chain ends. The α-keratins are part of the nails, horns, and hooves of mammals (hard keratin which contains 18–22% cysteine), hair, wool, skin (soft keratin, contains up to 14% cysteine) [1, 7];

2) β-keratins are harder and shaped as several zigzag-like polypeptide chains (β-folded sheets), usually anti-parallel to each other, which is stabilized by hydrogen bonds and hydrophobic interactions. These proteins contain much less cysteine, but are rich in amino acids such as glycine, alanine, and serine (a characteristic repeat of the "GSGAGA" sequence). Unlike α-keratin, the transverse disulfide bonds are absent between adjacent polypeptide chains of β-keratin, and the peptide fibrils are more flexible but not elastic. These proteins are found in feathers, beaks and claws of birds, in porcupine needles, in claws and scales of reptiles, in turtle shells and arthropod exoskeletons. The molecular mass of β-keratin varies from 10 to 22 kDa [1, 8, 9] (Fig. 1).

The food industries, especially the meat market, slaughterhouse and wool industry, produce millions of tones of biomass that contains keratin [1, 10]. Millions of tons of feathers are emitted into the environment as a by-product of the poultry processing enterprises, which makes serious problems as a pollutant, as well as sources of the H5N1 virus [11]. The human population constantly increases on the planet causing the intensive development of these industries, which, in turn, causes a steady increase in the amount of keratin-containing waste. These figures are over 40 million tons per year for countries such as the USA, Brazil, and China. Today, one of the main methods of utilization of these wastes is their burning. However, the method has a number of shortcomings: it is considerably costly, and burning the waste releases a significant amount of harmful gases in the atmosphere, which is dangerous to the environment [1].

Another method of utilizing feathers is the chemical treatment under pressure, which reduces their stiffness and increases digestibility. This method, however, is also highly expensive, and involves a breakdown of thermosensitive amino acids tryptophan and lysine [11]. Therefore, it is nowadays very important to develop a highly efficient and cheap way of processing keratin-containing raw materials. Alternatively, this issue can be solved by the enzyme biodegradation, which not only improves the nutritional value of feathers, but also offers mild conditions for the production of valuable products. Microbial keratinases (E.C. 3.4.21 / 24 / 99.11) are a group of proteolytic enzymes that meet these requirements because they are capable of decomposing complex and rich keratin waste into more easily digestible components [1, 11, 12]. The ability to synthesize these enzymes is found in some insects (moth larvae), as well as in microorganisms including bacteria, fungi, and yeasts, which can be isolated from keratin-containing waste [2, 3, 11, 13].

It has been found that the keratin-containing by-products contain 15–18% nitrogen, 2–5% sulfur, 3.2% minerals, 1.27%

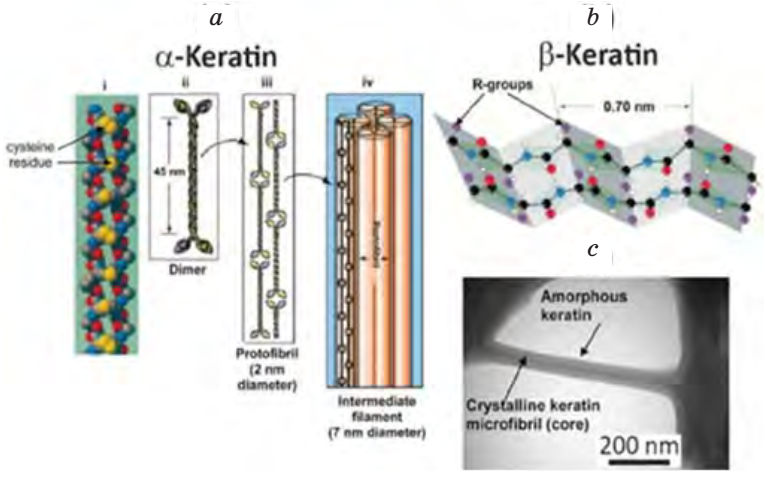

Fig. 1. **Structure of keratin:** *a* —Hierarchy of α-keratin showing the assembly from: two polypeptide chains (*i*) to a fibrous structure (iv); *b* — β-keratin with a pleated sheet shape that consists of antiparallel chains with R-groups that extend between sheets; *c* — TEM micrograph of α-keratin from a sheep horn displaying the composite structure of a crystalline keratin core within an amorphous keratin matrix [9]

fats and 90% protein. Hence, the rich in keratin organic waste can be a natural source of protein. Natural keratin, obtained from biomass, does not contain harmful chemicals and can be directly used for the production of various cosmetics, creams, shampoos, hair conditioners and biomedical products. Monomer units of natural keratin, penetrating the skin and cuticle of hair, can nourish them without any side effects [1].

Keratinases from microorganisms are also used to make protein supplements, animal feeds, skin treatments, and in detergents. And the keratinase produced by the action of cleavage products used in the production of nitrogen fertilizers, glue and foil, biofilms, as well as plastics [1, 10, 11, 14]. In addition, the promising direction is using keratinases in the degradation of prions for the treatment of bovine spongiform encephalopathy in order to prevent the prion-containing waste from contaminating the environment [5, 10, 15]. Keratinases can also be used as an active component of pesticides to combat root nematodes that cause the formation of galls, thickenings at the roots of plants [16, 17].

Consequently, the growing interest in microbial keratinases in various industries leads to the search for new keratinolytic producers of enzymes with properties that are commensurate with commercial needs.

Keratinase classification. Proteases are widespread, being involved in many biological reactions occurring both in the cell and in the body as a whole, and they play an important role in the circulation of nitrogen in nature. Their general action mechanism is that of the hydrolytic enzymes cleaving peptide bonds from the ends of the polypeptide chain (exo-protease) or within the chain (endo-proteases) [5]. According to the amino acid sequence in the active centers of enzymes and the catalytic action mechanism associated with it, these enzymes are grouped into asparagine, cysteine, glutamine, aspartate, metal, serine, threonine, mixed proteases, and those with an unknown catalytic action mechanism [14].

In the Enzyme Nomenclature (1979), the keratinases of *Streptomyces* and *Trichophyton* were named as E.C. 3.4.99.11 and 3.4.99.12 according to the type of catalyzed reaction [12, 13]. However, the intense development of molecular biology caused the improvement of the classification system. Based on the similarity of the amino acid sequences, which also reflects the evolutionary interactions between proteolytic enzymes, the MEROPS database united the proteases into 62 clans and 264 families now (http://merops.sanger.ac.uk/cgi-bin/family_index?type=P#S) [5, 14]. The classification of keratinases according to the MEROPS database is given in Table.

The authors [18, 19] classified the keratinase of *Meiothermus taiwanensis* WR-220 and the islandizin enzyme synthesized by *Fervidobacterium islandicum*, as belonging to the clan SB, family S8.

Depending on the nature of the active center, microbial keratinases can be serine proteases, metal proteases and serine metal proteases. The exception is yeast keratinases, which are asparagine proteases [14].

Metal and serine peptidases are endoproteases that cleave peptide bonds inside the polypeptide chain. Serine proteases are a functionally rich and diverse group of proteolytic enzymes with nucleophilic serine residues (Ser) in the active center. The latter attacks the carbonyl part of the peptide bond to form an intermediate compound (acyl-enzyme intermediate). To date, there are more than 333000 serine proteases that are classified in 53 families and 16 clans. Based on the structure, serine proteases are divided into two categories: trypsin-like and subtilisin-like. The enzymes of subtilisin subfamily are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and chymostatin [14].

In metal proteases, a nucleophilic attack on the peptide bond can be transmitted through the water molecules coordinated by the two-valent metal ion (usually Zn (II), sometimes Co (II), Mn (II)) or the bimetallic center of the enzyme (two ions Zn (II) or one Zn (II) ion and one Co (II) or Mn (II) ion). Depending on the amount of metal ions required for the catalysis, metalloproteases are divided into two groups: the first group requires two metal ions, and the other one needs only one. To date, there are about 294000 metalloproteases, united in 73 families and 15 clans [5]. Catalytic activity of metalloproteases is suppressed by chelating agents and heavy metals [14].

The microbial keratinases of *Actinomadura keratinilytica* Cpt29 [12, 20], *Streptomyces* sp. 1382 [21], *Actinomadura viridilutea* DZ50 [22], *Purpureocillium lilacinum* [23], *Aspergillus parasiticus* [24], *Bacillus pumilus* GRK [25], *Bacillus licheniformis* RPk [26], *Thermoactinomyces* sp. YT06 [27], *Brevibacillus brevis* [28], *Caldicoprobacter algeriensis* [29] are serine proteases.

Keratinases synthesized by *Acinetobacter* and *Bacillus subtilis* MTCC (9102) are metalloproteases [30, 31], while the enzyme of *Bacillus parabrevis* is a serine-metalloprotease [32].

Keratinases classification (MEROPS)

Name	Main name	MEROPS ID
Keratinase (*Bacillus* sp.)	Subtilisin Carlsberg	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.001; Catalytic type — serine; Subclass 3.4 (Peptidases) >> Sub-subclass 3.4.21 (Serine endopeptidases) >> Peptidase 3.4.21.62
Keratinase (*Doratomyces microsporus*)	Keratinase (*Doratomyces microsporus*)	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.148; Catalytic type — serine
Keratinase K1 (*Stenotrophomonas maltophilia*)	Keratinase K1 (*Stenotrophomonas maltophilia*)	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.110; Catalytic type — serine
Keratinase K2 (*Stenotrophomonas maltophilia*)	Subfamily S8A unassigned peptidases	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.UPA; Catalytic type — serine
Keratinase Ker P (*Pseudomonas aeruginosa*)	Pseudolysin	Clan MA >> Subclan MA(E) >> Family M4 >> Subfamily (none) >> M04.005; Catalytic type — metallo; Subclass 3.4 (Peptidases) >> Sub-subclass 3.4.24 (Metalloendopeptidases) >> Peptidase 3.4.24.26
Keratinase KerSMF (*Stenotrophomonas maltophilia*)	Subfamily S8A unassigned peptidases	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.UPA; Catalytic type — serine
Keratinase Sfp2	SFase-2 endopeptidase	Clan PA >> Subclan PA(S) >> Family S1 >> Subfamily E >> S01.431; Catalytic type — serine
Keratinase *Streptomyces albidoflavus*	Subfamily S1A unassigned peptidases	Clan PA >> Subclan PA(S) >> Family S1 >> Subfamily A >> S01.UPA; Catalytic type — serine
KerSMD (*Stenotrophomonas maltophilia*)	Keratinase K1 (*Stenotrophomonas maltophilia*)	Clan SB >> Subclan (none) >> Family S8 >> Subfamily A >> S08.110; Catalytic type — serine

Interestingly, *Bacillus halodurans* PPKS-2 synthesizes two keratinases which are different by the catalytic type: keratinase 1 is a disulfide reductase, keratinase 2 is a serine protease [33].

Consequently, most of the investigated keratinases are serine proteases, the synthesis of which is suppressed by phenylmethylsulfonyl fluoride (PMSF) and chymostatin [14].

Keratinase producers. Keratinolytic enzymes are synthesized by many bacteria, including actinomycetes, as well as fungi. Among the latter, a significant role is played by skin fungi, which in saprophytic state are able to digest keratin *in vitro* and use it as a substrate, and some can penetrate into the tissue (*in vivo*) and cause dermatomycosis in humans and animals [7].

There are two types of fungi that inhabit keratin substrates:

• keratinolytic fungi, which affect the keratin substrate directly and cleave the molecules;

• keratinophilic fungi, which use the matter that is naturally linked to keratin, or the products of keratin destruction under the effect of other fungi [7].

Keratinases are produced by both pathogenic and non-pathogenic fungi: *Alternaria*, *Arthrographis* [34], *Aspergillus* [24, 35], *Beauveria*, *Chrysosporium*, *Cladobotryum*, *Cladosporium* (*C. sphaerospermum*) [34], *Doratomyces* (*D. microsporus*) [3, 36], *Geomyces*, *Gymnoascus*, *Malbranchea*, *Microsporum*, *Mucor*, *Myceliophthora* [34], *Paecilomyces* (*P. marquandii*) [36], *Pectinotrichum* [34], *Penicillium* (*P. citrinum*) [37], *Purpureocillium* [23], *Renispora* [34], *Scopulariopsis* [7], *Sporendonema*, *Trichophyton* [34]. The pathogenicity and virulence of some fungi are closely related to the ability of the producers to break down both hard and soft types of keratin. However, only non-pathogenic keratinolytics are of an industrial importance, because of the risk of infection with pathogenic strains.

Among the Gram-positive bacteria, the ability to synthesize keratinases and cleave keratin is found in the genera *Arthrobacter* [38], *Bacillus* [12], *Kocuria* [39], *Lysobacter* [12], *Microbacterium* [8], *Nesternokia* [12], etc. However the most common producers of keratinases are the representatives of

the genus *Bacillus*, namely *B. subtilis* and *B. licheniformis* [26]. Although keratinolysis is detected in other species of the genus *Bacillus*: *B. altitudinis* [40], *B. halodurans* [33], *B. tequilensis* [41], *B. pumilus* [25], *B. cereus*, and *B. thuringiensis* [42]. Several genera of the Gram-negative bacteria also can synthesize keratinases: *Acinetobacter* [30], *Alcaligenes* [43], *Caldicoprobacter* [29], *Chryseobacterium*, *Fervidobacterium* [8, 14], *Klebsiella* [44], *Stenotrophomonas*, *Thermoanaerobacter* [8, 14, 45], *Vibrio*, *Xanthomonas* [8].

The keratinolytic activity is also found in several actinomycetes: *Actinomadura* (*A. keratinilytica*, *A. viridilutea*) [20, 22], *Nocardiopsis* [10], *Streptomyces pactum*, *S. graminofaciens*, *S. albidflavus*, *S. flavis* [10].

Recently, researchers became interested in thermophilic and hyperthermophilic organisms that synthesize keratinases with unique properties. The temperature optimum of those enzymes is within the range of 80–100 °C: *Aeropyrum*, *Fervidobacterium* [46], *Microbisporaaerata* [10], *Pyrococcus* [46], *Streptomyces gulbarguensis*, *S. thermoviolaceus*, *S. thermonitrificans* [10], *Thermoactinomyces* [27], *Thermoanaerobacter*, *Thermococcus* [46], etc. These enzymes are promising for research, since they can be used in high-temperature processing of keratin-containing raw materials.

Keratinase synthesis regulation. Microbial keratinases are predominantly extracellular enzymes that are synthesized in the culture medium containing keratin as an inducer [4, 22, 41, 47]. Although there are microorganisms capable of producing intracellular keratinase, in particular *Trichophyton gallinae* [48]. The geophilic microscopis fungi *Arthroderma quadrifidum*, *A. curreyi* and *Chrysosporium pruinosum* can synthesize both extracellular and cellular keratinases [14].

The microbial production of enzymes is a complicated and highly regulated process that depends on the growth phase of the microorganism. Production of keratinase is most intense at the end of the exponential or in the stationary phase of growth [5, 11, 49].

Most keratinases are inducible enzymes, although some are expressed constitutively [5, 14]. Adding keratin or keratin-containing substances in the culture medium is important for the induction of keratinase synthesis. The most common source of carbon and nitrogen is chicken feathers [20, 25, 39], both alone and in combination with yeast extract [26], a mixture of chicken feathers and keratin [24, 38], hair keratin [37]. However, non-keratin substrates such as soy flour [47], starch [49], sucrose [27], and dextrose [50] can also induce the synthesis of these enzymes. If glucose is used as a carbon source, the synthesis of certain keratinases is suppressed, which is associated with the phenomenon of catabolite repression [8, 47]. However, there is no repression of the keratinase synthesis in *A. parasiticus* enzyme in the presence of a mixture of 1% glucose with 1% keratin [24], similarly to the keratinase of *B. thuringiensis* Bt407 [49].

The keratinase synthesis is also influenced by the presence of organic or inorganic nitrogen sources. Keratin-containing substrates may serve as a source of nitrogen [25]. Still, researchers sometimes use extracts of yeast extract [24, 26, 49] or a combination of inorganic nitrogen (potassium nitrate [38], sodium nitrate [27]) or peptone [50, 51] or tryptone to extract a significant amount of enzyme [51].

Another important factor influencing the production of enzymes, in addition to the content of nutrient medium, is the cultivation conditions: the optimum growth temperature of the producer, the initial pH value of the medium, the intensity of mixing and the amount of seed material [8, 14].

Temperature is the determining factor in the synthesis of enzymes. However, the optimum synthesis temperature depends on whether the culture is mesophilic or thermophilic. Most keratinolytic fungi are mesophiles producing keratinases at temperatures 26 °C [37], 28 °C [23], or 30 °C [24, 35]. Bacterial keratinases are usually synthesized at a temperature of 25 °C [39], 37 °C [25, 26, 33, 38, 50], 50 °C [29]. However, there are thermophilic and hyperthermophilic bacteria *Thermoanaerobacter* and *Fervidobacterium* that secrete keratinases in the medium at elevated temperatures, 65 °C [52] and 70 °C [12], respectively.

Also an equally important parameter in the synthesis of keratinases is the initial pH of the nutrient medium. Most of investigated keratinases were produced at neutral [38, 49, 50, 51] or alkaline [25, 27, 33, 51] pH values. Although the fungi *P. marquandii*, *D. microsporus* [36], *A. parasiticus* [24] and bacterium *B. subtilis* KD-N2 [53] synthesized keratinases in weakly acidic media with pH values of 6.0 and 6.5, respectively.

The amount of inoculum introduced into the nutrient medium is an important factor affecting cell growth and the formation of the target product. The amount of seed material used in different studies usually varies from

1% to 5% [24, 25, 38]. Additionally, if inoculum portion was up to 10%, that was optimal for the synthesis of keratinase by *B. subtilis* KD-N2 in a hair-containing medium [53], while 50% inoculum was needed for the keratinase synthesis by *B. subtilis* MTSC9102 [50] on horns as a substrate at solid phase culturing.

Most microbial producers of enzymes are aerobic and need oxygen for their growth and development. Lack of oxygen can lead to adverse changes in the enzyme composition and loss of the target product, or even kill the organism. Oxygen requirements during enzymatic processes are provided by aeration and mixing [8]. The degree of aeration for the synthesis of keratinase is: 100 rpm [36], 120 rpm [49], 140 rpm [35], 150 rpm [38, 51], 180 rpm [27], 200 rpm [23, 25, 26], 220 rpm [37].

Microbial production of keratinases is carried out by submerged (SmF) [8, 29, 54] or solid -state fermentation (SSF) [50, 55, 56]. Using SPA has several advantages over SmF: the enzymes are cheaper, the minimum energy costs, the stability of output product, the lower cost of water, and better oxygen circulation [8]. The SmF method, in contrast to SSF, makes it possible to automate the process parameters needed for the optimum growth.

Thus, the selection of the appropriate nutrient medium and cultivation conditions can not only increase the yield of enzymes, but also produce preparations with certain properties. Thus, the authors [57] investigated ten microbial isolates on their ability to produce keratinases. The most active isolate, identified as *B. licheniformis* ALW1, exhibited 25.2 U/ml keratinase activity. After the optimization of cultivation conditions, the biosynthesis of keratinase increased almost three times to 72.2 U/ml.

Fungal producer *Aspergillus* sp. DHE7 [11] was selected among the 15 keratinolytic strains isolated from the poultry farm soil. It showed the highest keratinase yield of 199 ± 4.2 U/ml when incubated for 4 days at 30 °C and pH 6.0 on a 2% chicken feathers substrate. The addition of 0.5% sucrose as a supporting source of carbon increased the keratinase yield to 226 ± 5.4 U/ml, while introducing the additional sources of nitrogen to the medium did not affect the enzyme synthesis. The authors [11] have shown that the best substrates for the keratinases synthesis are goat hair (452 ± 12.3 U/ml), turkey feathers (435 ± 9.2 U/ml) and sheep wool (322 ± 13.4 U/ml). The obtained results promote using *Aspergillus* sp. DHE7 in the environmentally friendly process of bioconversion of keratin waste.

Quite often, keratinase genes are cloned in host cells in order to increase the enzyme output, which is necessary for the its commercialization. Thus, more than 50% of industrially important keratinases are produced by heterologous expression in the host cells, which are specially adapted for the intensive production of target enzymes. *E. coli* is commonly used as an expression system for recombinant proteins [58]; however, the limiting factors in obtaining the enzyme using that host are the accumulation of inactive compounds [59], as well as the need for folding of pro-keratinase *in vitro*, which essentially affects the yield of the final active product. If expression in the host cells does not provide the proper folding of the proteins, additional procedures are needed to form disulfide bonds [5]. Therefore, other microorganisms, including representatives of different species of the genus *Bacillus* can be used as host cells [60], in which the introduction and expression of numerous copies of keratinase genes leads to the increased enzyme output. However, the use of those producers may be limited by plasmid instability. In addition, certain *Bacillus* species synthesize a lot of other enzymes inherent in that producer (amylase, mannanase, cellulase), which can negatively affect the process of separation and purification of the target product [5]. Quite often, *Pichia pastori*s yeasts are used as an expression system for the production of keratinases, providing an appropriate medium for posttranslational modifications and folding of eukaryotic keratinases [59]. These single-celled eukaryotes are easily cultured and manipulated, so they are successfully used to expression many proteases of bacteria, fungi and mammals [5].

Physical and chemical factors are also used to obtain mutants with elevated keratinase activity. Thus, a mutant *Streptomyces radiopugnans* KR 12 strain was obtained as a result of UV irradiation. Its keratinolytic activity was thrice increased compared to wild strain [61].

Chemical agents such as ethyl-methylsulfonate (EMC), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG), or ethidium bromide, can also be used to induce random mutations in the DNA molecule [5]. For example, the use of MNNG resulted in the production of a mutant *B. subtilis* strain KD-N2, whose keratinase activity was 2.5 times greater than that of wild strain [62].

Four amino acid substitutions (N122Y, N217S, A193P, N160C) were introduced into the keratinase of *B. licheniformis* BBE11-1 using site-directed mutagenesis, which made it possible to increase its catalytic activity by 5.6 times in comparison with wild-type keratinase [63].

Physical and chemical properties of keratinases

Molecular weight of keratinases. Most keratinases isolated from bacteria, fungi, and actinomycetes are monomeric enzymes with molecular weight varies from 14 to 240 kDa [12, 14, 29]. The lowest molecular weights of 15–20 kDa and 18 kDa are characteristic of the *Streptomyces* sp. 1382 [21] and *Streptomyces albidoflavus* [5, 64] keratinases, respectively. Keratinases isolated from bacteria, including actinomycetes, are characterized by the following molecular weight ratios: 43 kD in *B. altitudinis* RBDV1 [40], 30 and 66 kDa in *B. halodurans* PPKS-2 [33], 32 kDa in *B. licheniformis* RPk [26], 28 kDa in *B. tequilensis* Q7 [41], 28 kDa in *Brevibacillus parabrevis* [32], 25 kDa in *Acinetobacter* [30], 33 kDa in *Caldicoprobacter algeriensis* [29], 19.5 kDa in *A. viridilutea* DZ50 [22], 29 kDa in *A. keratinilytica* Cpt29 [20], 35 kDa in *Thermoactinomyces* sp. YT06 [27]. Four fractions with keratinase activity have been isolated from *Micrococcus luteus* with the following molecular weights: 62 kDa, 139 kDa, 185 kDa and 229 kDa, respectively [52]. The highest molecular weight, 240 kDa, is found for the keratinase of *Kocuria rosea* [14]. High molecular weights are usually characteristic of keratinases synthesized by thermophiles or which are metalloproteases [29, 36, 53]. Thus, an anaerobic thermophilic bacterium *Thermoanaerobacter* sp. 1004-09, isolated from hot springs of the rift zone of Baikal, synthesizes keratinase with a molecular weight of 150 kDa [53].

Some bacterial producers of keratinases synthesize multimeric proteins [14, 29]. For example, *B. licheniformis* ER-15 keratinase is a dimeric enzyme with subunits of 28 and 30 kDa [65]. And three keratinolytic enzymes (K1, K2 and K3) of 48 kDa, 36 kDa and 17 kDa, respectively, were obtained as a result of the 3-stage purification from *Stenotrophomonas maltophilia* BBE11-1 [66].

Fungal keratinases usually have low molecular weights of 20 to 40 kDa: 21 kDa in *P. citrinum* [54], 37 kDa in *Purpureocillium lilacinum* [23], 36 kDa in *A. parasiticus* [35], 33 kDa in *P. marquandii*, and 30 kDa in *D. microsporus* [36]. Although it is noted that keratinases synthesized by pathogenic fungi (for example, keratinase 2 of *Trichophyton mentagrophytes*) have a molecular weight of up to 440 kDa [5].

pH- and thermal optimum, pH- and thermal stability of keratinases

Thermal optimum, pH optimum, as well as thermostability of enzymes are important characteristics that determine the possibility of their further application.

Most of microbial keratinases achieve the highest activity in the alkaline or neutral pH values of 7.0–9.0: keratinase of *B. altitudinis* RBDV1 [40], *B. parabrevis* [32], *B. brevis* [28], *Bacillus* sp. P45 [67], *Bacillus thuringiensis* Bt407 [49] at pH 8.0; keratinase of *B. tequilensis* Q7 [41], *C. algeriensis* [29], *A. parasiticus* [35] at pH 7.0; keratinase of *B. licheniformis* RPk [26] at pH 9.0. However, some enzymes are optimally active outside this values, even at extreme alkaline or slightly acidic pH [29]. Thus, keratinase of *B. subtilis* MTCC (9102) exhibits maximum activity in a weakly acidic pH of 6.0 [31]. Several keratinolytic enzymes are notably stable in a wide range of pH [23, 29, 52, 60]. The enzyme of *A. keratinilytica* Cpt29 is active at pH of 3.0 to 10.0, with the optimum pH of 10.0 [20]; keratinases obtained from *B. halodurans* PPKS-2 are active at pH of 7.0-13.0 with the optimum pH of 11.0 [33]. The protease synthesized by *Streptomyces* sp. AB1 is stable at pH 4.0–11.0 for 96 hours with the optimum pH of 11.5 [68]. The maximum activity at a pH of 11.0 was also detected by keratinases of *A. viridilutea* DZ50 [22], *Acinetobacter* [30], *B. licheniformis* ER-15 [65]. The pH of 10.0–10.5 was optimum for keratinases, synthesized by the recombinant strain *B. subtilis* WB600 [60] and *Meiothermus taiwanensis* WR-220 [18]. The highest value of the pH optimum in the alkaline region of 12.5 is characteristic for keratinase of *Nocardiopsis* sp. TOA-1 [69].

The optimum temperature of keratinase activity is very diverse and often depends on the producer and temperature conditions of the producer growth [29, 38, 60, 64]. Quite often, though, the maximum activity of an enzyme is observed at a temperature higher than the optimum conditions for the growth of a microorganism [26, 33, 35]. Thus, the keratinase of *A. viridilutea* DZ50 exhibits maximum activity at a temperature of 80 °C, while the producer growth temperature is 45 °C [22]; enzyme synthesized by *Nocardiopsis* sp. TOA-1, which grows at 30 °C, has a temperature optimum at 60 °C [69].

Enzymes of thermophilic microorganisms *A. keratinilytica* Cpt29, *Streptomyces* sp. strain AB1, *A. viridilutea* DZ50, *B. altitudinis* RBDV1 have a temperature optimum at 70 °C [20], 75 °C [68], 80 °C [22], and 85 °C [40], respectively, whereas mesophilic cultures exhibit maximum activity at 40 °C [28, 31, 38, 60]. The lowest optimum temperature of 30 °C is characteristic for enzymes synthesized by *B. tequilensis* Q7 [41] and *P. citrinum* [54] and the highest for keratinases of hyperthermophilic microorganisms isolated from marine hydrothermal sources that exhibited maximum activity at a temperature of 100 °C and above [18, 46].

Many keratinases exhibit maximum activity at a temperature of 50–55 °C, particularly those of *M. luteus* [70], *A. parasiticus* [35], *Acinetobacter* [30], *C. algeriensis* [29], *Bacillus* sp. P45 [67], *B. thuringiensis* Bt407 [49]. Certain enzymes exhibited thermal optimum at a temperature of 60 °C, such as produced by *B. licheniformis* RPk [26], *B. parabrevis* [32], *Thermoanaerobacter* 1004-09 [52], *Nocardiopsis* sp. TOA-1 [69]. Keratinases, active at high temperatures, are used in various industries and in medicine.

The enzyme thermostability is an important characteristic for most biotechnological processes. Hence, thermophilic and hyperthermophilic microorganisms are of particular interest as a potential source of new thermostable enzymes. There are several advantages in using thermostable enzymes in industrial processes: reducing the risk of microbial contamination, reducing the viscosity and mixing speed, increasing the solubility of the substrate and the diffusion rate [19, 46].

The thermal activity and thermal stability of most keratinases is found to increase in the presence of Ca^{2+} ions [13, 26, 28, 49], and those of several other enzymes in the presence of Mg^{2+} [13, 40, 71] or Mn^{2+} ions [20, 40, 70]. Interestingly, Ca^{2+} ions did not affect the activity of keratinase *Thermoanaerobacter* sp. 1004-09, but significantly increased its thermal stability from 10 min to 13 h at a temperature of 94 °C [52].

The keratinase of *Meiothermus taiwanensis* WR-220 is known to contain two calcium binding sites [18] (Fig. 2). The elimination of Ca^{2+} ions from such site causes a significant reduction in the thermostability of enzymes. The role of Ca^{2+} ions may be related to the stabilization of the activated keratinase form and the protection of its structure from autolysis [29, 49].

There also are thermolabine keratinases. For example, keratinase of *Bacillus* sp. P45 showed maximum activity at 55 °C, but it had low thermal stability and was completely inactivated after 10 min at 50 °C [67].

Investigating the influence of temperature on the activity of various keratinases, it was shown that they exhibit activity both in the wide [18, 20, 31, 40, 52, 60, 68], and narrow values of temperatures [54].

Substrate specificity. Microbial keratinases isolated from different sources are characterized by different properties depending on the producer. Keratinases synthesized by fungi, actinomycetes, and bacteria are capable of cleaving a wide range of substrates from soft keratin (e.g., corneous layer [65]) to hard keratin of feathers [20, 27, 39, 43, 44, 65, 66, 70], wool [2, 30, 32], human hair [36] and animal hair, hoof [66], horn [13], azokeratin [68], keratin azure [28, 67, 68, 70]. Also, certain keratinases degrade collagen [13, 71], elastin [28, 59], gelatin [13, 28, 60, 70], albumin [28, 60, 71], hemoglobin [28, 65], fibrin [65], and azocasein [28, 35].

Keratinase substrate specificity depends on the chemical properties of the substrates. Since keratin consists of 50–60% hydrophobic and aromatic amino acids, keratinases predominantly cleave peptide bonds containing P1 hydrophobic and aromatic amino acid residues in P1 position [4, 59]. The study of hydrolysis using an oxidized insulin B-chain as a substrate showed that the keratinases of *Thermoanaerobacter* sp. 1004-09 and *P. aeruginosa* KP1 and KP2 selectively hydrolyze the bonds formed by phenylalanine, valine, tyrosine or leucine [72]. Substrate specificity of these keratinases was determined using synthetic substrates, such amino acid derivatives as *p*-nitroanilides (pNA), *p*-nitrophenyl esters (ONp), and derivatives of 7-amino-4-methylcumarin (AMC). The residues in the P2 and P3 positions also play a role. The keratinase KerS14 of *B. subtilis* predominantly cleaves peptide bonds with Arg in position P1, Gly and Ala in position P2, Gln or Glu at position P3 [14].

Study of the ability of *R. marquandii* and *D. microsporus* keratinases to hydrolyze various substrates showed that the enzymes cleaved α-keratin of skin and nails, to a lesser extent that of hair and wool, but could not cleave chicken feathers β-keratin. It was established that the presence of reducing agents stimulated the enzymatic hydrolysis of keratin. When 1mM dithiothreitol was added, the keratinase activity of *R. marquandii* and

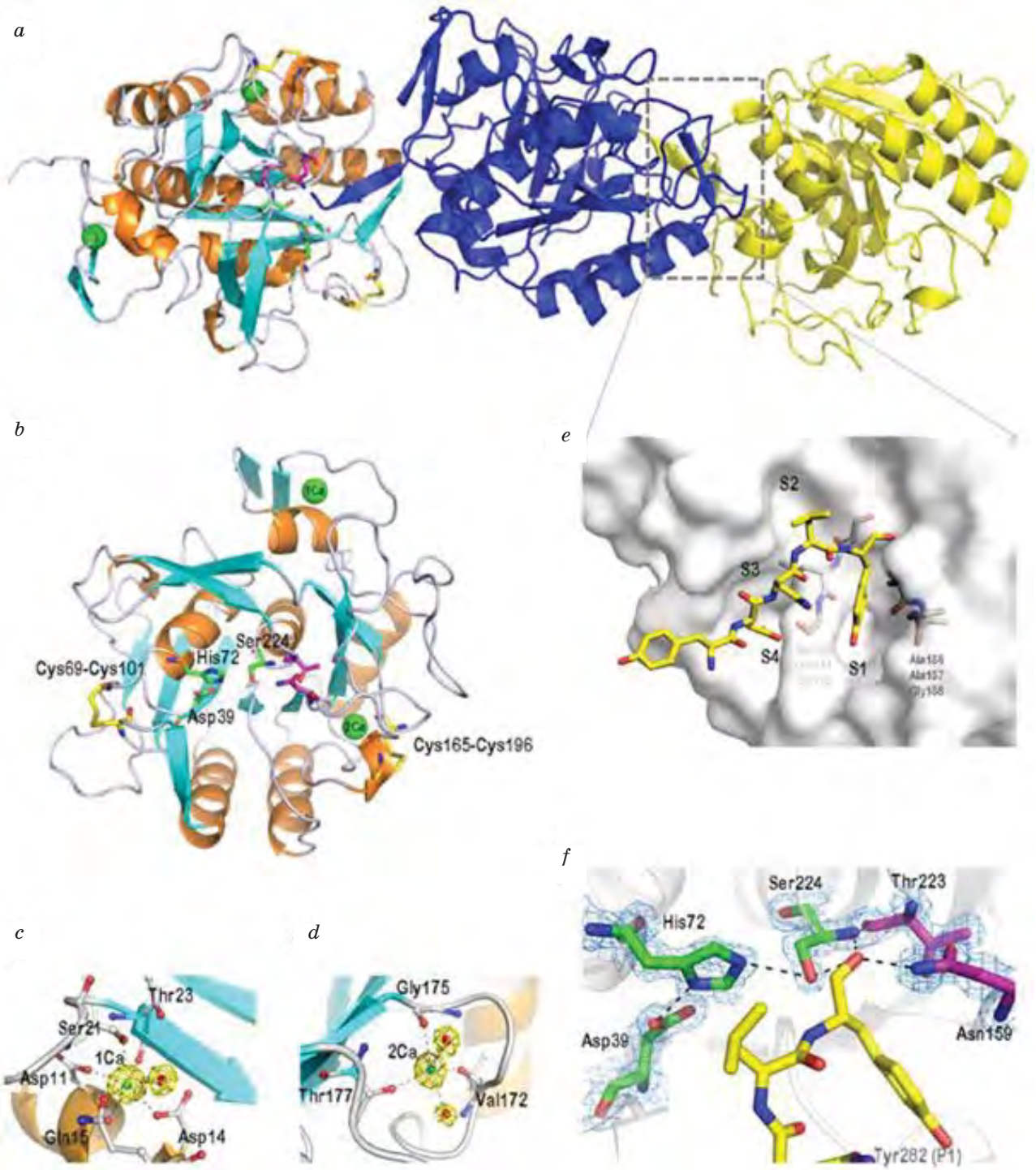

Fig. 2. **Crystal structure of rMtaKer:**
a — Stereoview of three mature rMtaKer monomers is shown in the asymmetric unit. The trimers are named chain A (orange/cyan), chain B (blue), and chain C (yellow); *b* — The core structure of mature rMtaKer is made of a seven-stranded parallel β sheet flanked by six α-helices (orange) and five β-sheets (cyan). The calcium ions are shown as green spheres and two intramolecular disulfide bridges are highlighted in yellow. The catalytic triad (Asp, His, and Ser) is labeled by green sticks and the oxyanion hole residues are marked by magenta sticks; *c* — Structure of the 1Ca-binding site forms a pentagonal bipyramidal geometry with five residues and one water molecule (red ball); *d* — Structure of the second Ca-binding site is coordinated to three residues and two water molecules; ***e*** — The end part of the C-terminus (Tyr-Glu-Asn-Leu-Tyr) from C chain binds at the substrate-binding cleft (S1–S4) on the B chain of rMtaKer as shown in gray. The substrate is displayed as a yellow stick model; *f* — Tyr282 seals the hydrophobic pocket of S1 site and several hydrogen bonds are also observed along the pocket surfaces and around the active site [18]

D. microsporus increased two and three times, respectively [36]. This is due to the ability of reducing agents to decrease the amount of disulfide bonds in keratin threads, thereby promoting the access of enzymes to the substrate for proteolytic attack [36].

Keratinase produced by *P. citrinum* PC-54-91 VILAR cleaves α-keratin and almost does not hydrolyze collagen [54].

Keratinase, synthesized by *B. pumilus* FH9, showed broad substrate specificity, splitting both soluble and insoluble substrates. The enzyme showed the highest proteolytic activity on casein, serum bovine albumin, gelatin, collagen, and to a lesser degree was able to hydrolyze feathers, wool and horns [13].

Hence, there are keratinases with a narrow, and with a broad substrate specificity [13, 28, 66, 70].

Practical use of keratinases. Microbial enzymes make up a significant proportion of industrial catalysts, of which about 65% of the market is occupied by hydrolases. Among the latter, there is a very important group of proteases with a wide range of applications [5]. The increased commercial interest in microbial enzymes is due to the fact that microorganisms are their inexhaustible source due to their natural diversity, ease of cultivation, safety at work and the ability to genetically change. In addition, microbial enzymes also have many advantages over chemical compounds and animal enzymes, such as high activity, broad substrate specificity, and ability to biodegrade [1, 4, 10].

Keratinases, due to their broad substrate specificity and the ability to split the substrate resistant to hydrolysis, are used in many industrial processes (Fig. 3).

Annually, a very large amount of keratin waste is formed worldwide. The main environmental pollutants are poultry, leather and textile industries, and a lot of waste in the form of feathers, hair, bristles, horns and hooves is obtained from livestock and slaughterhouses [14, 17, 45].

However, there are some restrictions on the use of keratin waste in the European Union (EU). Following the outbreak of bovine spongiform encephalopathy (BSE) in the United Kingdom, the European Union and the United States of America introduced strict rules regarding the use of animal by-products, namely grouping them into three categories in accordance with the level of risk of transmission of pathogens and toxic substances. Only keratin waste of the third category can be processed and used for the production of feed.

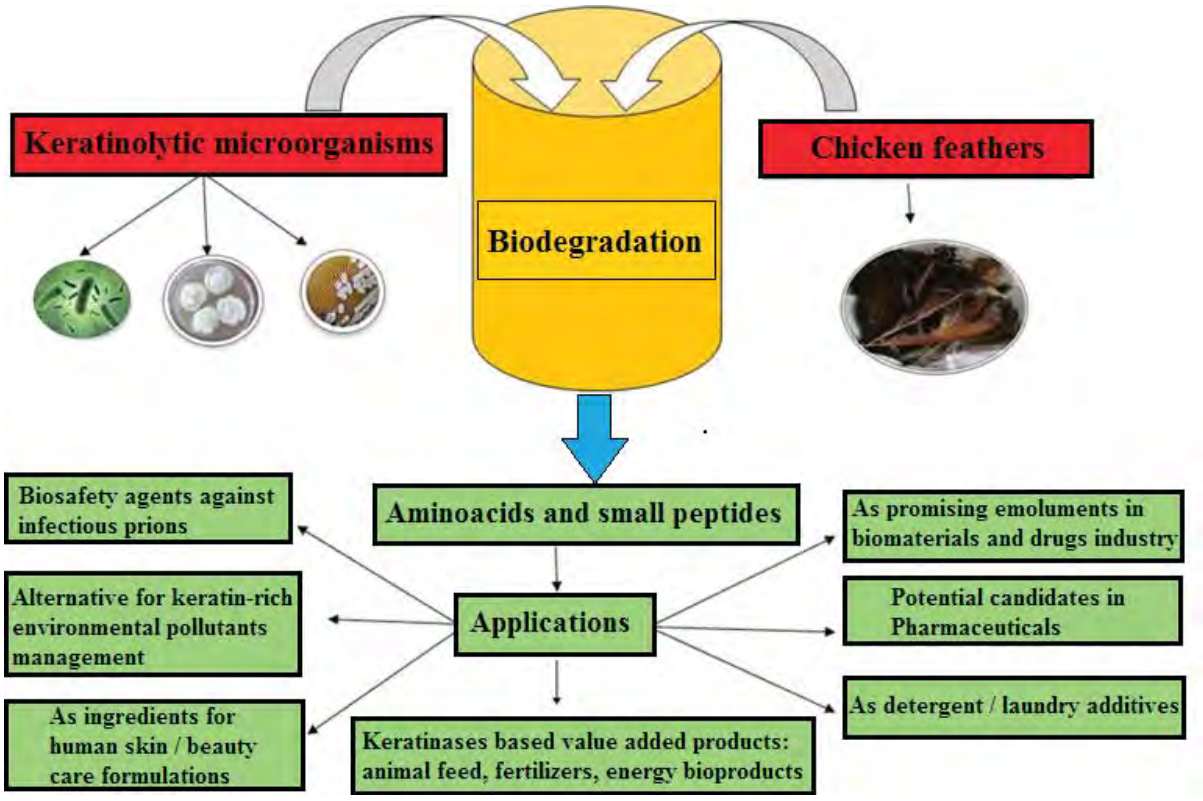

Fig. 3. **Schematic representation of current and potential applications of microbial keratinases [10]**

According to the Regulation (EC) No 1774/2002 of the European parliament, the third waste category includes the products of animal origin that 1) are obtained from animal carcasses, 2) are not used for human consumption, 3) will not transmit pathogens to humans and animals. A large amount of waste produced by animal processing plants can be used as a substrate for bioenergy and high-value products if these wastes are pre-treated properly [14].

In 2012, around 8.5 billion tons of bird feathers were produced worldwide [5]. Bird feathers consist of keratin (predominantly β-keratin) and contain a significant amount of serine, glutamic acid, proline and a small amount of methionine, histidine and lysine. One of the ways to process feather waste is transforming them into flour which can then be used as raw material in the production of biofuel, as an ingredient of bioplastics and as a feed for animals. The traditional method of treating feathers involves high temperature and pressure, is energy-intensive and causes the loss of several essential amino acids (methionine, lysine, histidine and tryptophan). An alternative method is to treat feathers with keratinases and obtain hydrolysates, which, in their nutritional value and digestibility, exceed the products obtained by chemical and mechanical processing [3, 5, 14, 46]. High-quality amino acids thus obtained can be added to feed birds, ruminants, pigs and fish [5, 47].

Another way to dispose of keratin waste is composting, during which organic keratin waste is gradually converted into inorganic nitrogen (ammonium and nitrate) and sulfur (sulfate), which can then be easily absorbed by plants [14]. However, this process is very long-term because of the stability of the substrate to the action of most proteolytic enzymes. At first, within one to four weeks, bacteria and actinomycetes develop within the compost and are gradually replaced by fungi. Keratinolytic strains are detected around the sixth week, their growth correlates with mineralization of nitrogen and sulfur. To accelerate and intensify the process of composting, it is possible to inoculate compost with keratinases of microorganisms [14]. Various studies have shown that the addition of *B. licheniformis* and *Streptomyces* sp. greatly improved the degradation of chicken feathers compost and obtaining valuable products that can later be used in agriculture [14].

In laboratory conditions, many microorganisms were shown to break down chicken feathers and other keratin-containing products [20, 28, 44, 55, 65, 67].

BioResource International (BRI) produces enzyme preparations Versazyme® and Valkerase® containing keratinases of *B. licheniformis* and used to degrade the keratin waste [72].

The hydrolysed feathers can also be used to produce biohydrogen or as fertilizers in organic farming promoting the slow release of nitrogen, improving plant growth, and enhancing the activity of microorganisms in the soil, structuring it, and also increasing the moisture-retaining capacity of the soil [5].

Thus, hydrolyzates of bovine horns and hooves produced using *P. marquandii* can be utilized as fertilizers because they contain a significant amount of amino acids (with the exception of proline and tryptophan), and significantly differ from other fertilizers by a positive effect on plant growth. The hydrolyzates obtained as a result of *P. woosongensis* TKB2 splitting the bird feathers contribute to seed germination and seedlings growth [14, 47].

The transformation of keratin waste into biofuel is a far-reaching direction for producing clean energy that can partially meet the global demand for energy. Feather hydrolyzate produced by *B. lichenifomis* keratinase can then be used by *Thermococcus litoralis* culture to obtain biogas [3, 14, 46].

The keratinases are most widely used in the leather industry, which is one of the oldest and fastest growing industries in the world, important in the modern economy. At the same time, it represents one of the world's largest sources of pollution, since skin treatment requires the use of toxic substances hazardous to the environment, including workers of such enterprises [5, 14, 42, 73]. The production of leather involves several stages, one of which is the removal of epidermis, hair, and wool. That is done with chemical reagents (alkali, sulfides) which destroy disulfide bonds in the molecules of structural proteins and sadly pollute the surrounding nature. The use of keratinases is an alternative solution (Fig. 4), which leads to a decrease in the level of environmental pollution, and improves the quality of the final product. The enzymes significantly reduce the time and cost of "dehairing", simplify the general scheme of leather treatment and the final product is of higher quality. Proteolytic enzymes are increasingly used to soften the leather and in preparing it to the tanning process. Keratinases can remove animal hair, but they should not exhibit collagenase activity in order not to damage the leather. The enzymes most

often used in the leather industry are produced by *Bacillus* sp., *Pseudomonas stutzeri*, *Cladicoprobacter algeriensisi*, *Acinetobacter* sp., *Paenibacillus woosongensis*, *Vibrio metschnikovii*, *Microbacterium* sp. kr10 and various fungal species: *Aspergillus tamarii*, *Penicillium chrysogenum* and *Trichoderma harzianum* [5, 14]. For example, the keratinase of *Bacillus safensis* LAU 13 fully dehairs the goat skin in 12 hours without obvious damage compared to the chemical method which does not allow for complete dehairing and involves leather damage [47]. The keratinase of *B. brevis* US575 effectively dehairs the skins of rabbits, sheep and cows [28].

At one of the stages of leather treatment, chromium sulphate ($CrSO_4$) is used to stabilize it. The compound is only partially bound and most of it gets into sewage. The permissible level of Cr in sewage in most countries of the world is less than 2 mg/l, so it is necessary to increase the absorption rate of Cr in the processing. Adding keratin hydrolyzate (2–3% w/w) of horn flour makes it possible to reduce Cr in sewage from 35% to 10%. The low molecular weight keratin peptides contained in the hydrolyzates react with Cr to form the Cr-keratin complex, which, when interacting with collagen, enhances absorption of Cr [14].

As part of complex proteolytic preparations, keratinases are used in soaking, liming and softening of skins. Patented special preparations for "dehairing" consist of a mixture of enzymes isolated from micromycetes, streptomycetes and bacteria of the genus *Bacillus* [37].

Keratinases, particularly those isolated from *B. licheniforms*, *B. cereus*, *Chryseobacterium* L99 and *Pseudomonas* sp., are important in the textile industry, since they can improve the fiber resistance to shrinkage and staining [47, 60]. Applying an enzyme complex of keratinase, cutinase, lipase and transglutaminase can greatly improve the process of wool processing [72].

The most promising direction is using keratinases in the detergent industry, because most of the enzymes are alkaline proteases stable at relatively high temperatures and sufficiently tolerant of surface active compounds [20, 25, 32, 35, 67]. Being substrate specific, keratinases are able to remove contamination over a short period of time without damaging the structure and strength of the fibers. They are used to hydrolyze the keratin derivatives on collars and cuffs [10, 14]. The alkaline keratinase of *P. woosongensis* TKB2 effectively removes blood

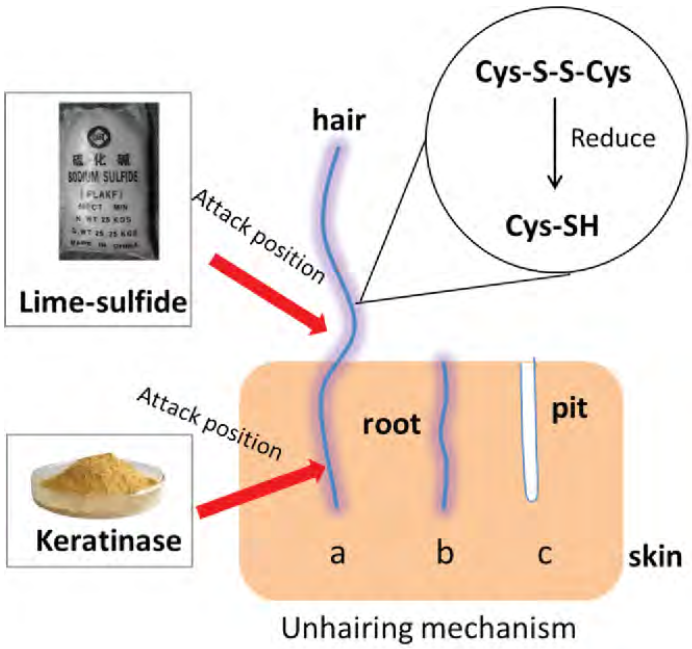

Fig. 4. **Two unhairing strategies (lime sulfide and keratinase) used in beamhouse process. Though those two strategies share similar unhairing mechanism of reducing S-S bond to dissolve hair protein, lime sulfide attacks the hair shaft outside the skin, while keratinolytic protease attacks the hair root to produce shaft free skins:**
a — whole hair in skin without treatment; *b* — unhairing with lime sulfide and hair shaft was still remaining in the hair pit; *c* — unhairing with keratinase and skins are free from hair shaft [73]

stains from surgical clothes, and egg yolk and chocolate from ordinary clothing. And *B. thuringiensis* TS2 keratinase degrades not only blood stains and egg yolk, but also effectively removes hair from goat skin [3].

Keratinases can also be applied in the detergent industry in the treatment of wastewater, generated by the laundry washing and containing a large amount of keratin waste and dirt [3, 5, 14]. The commercial product BioGuard Plu, manufactured by RuShay Inc., is used to clean drainage pipes and septic tanks [72].

Also, keratinases are widely used in the biomedical, pharmaceutical and cosmetic industries, in the preparation of vaccines, the production of bioactive peptides, therapeutic serums, the creation of cosmetic products (nutritional creams, lotions, anti-dandruff shampoos); callus removal, degradation of keratinized skin, its epilation, removal of keratin in the treatment of psoriasis and acne [3, 5, 10, 14, 72].

Proteos Biotech produces commercial preparations: Keratoclean® Hydra PB, Pure100 Keratinase, Keratoclean Sensitive PB and Keatopeel PB for callus removal and acne treatment [72].

Keratinases are used to cleave dead skin cells, improve blood circulation and thoroughly and deeply cleanse it, prepare skin for absorption of nutrients: masks, creams, as well as ampouled preparations and serums, which are extremely useful for dry and sensitive skin [1, 42].

The hair consists mainly of keratin (90%) and a small amount of lipids (1–9%). Keratin hydrolyzates are effective restorers in the hair care process. Most of the keratin hydrolysates used for this purpose are chemically hydrolyzed or thermally treated hooves, horns and wool, although recently using of microbial keratinases has become popular. Treatment of chicken feathers with keratinolytic enzymes of *S. maltophilia* resulted in hydrolyzates which had a positive effect on the hair, as evidenced by the strength, shine, softness of both normal and damaged hair [14, 46].

The two most common diseases for which keratinase are used are onychomycosis and psoriasis. The nail plate consists essentially of 80% of "hard" keratin and 20% of soft keratin. For effective local nail treatment, it is necessary to relax the hard nail plate keratin. For example, *P. marquandii* keratinase increases the delivery of preparations by partial hydrolysis of nail plates [14]. FixaFungus and Preteos Biotech produce FixaFungus ™ and Kernail-Soft PB, used to treat nails [72].

The keratinase ability to hydrolyse keratin can also be used to heal wounds. The avascular nature of the wounds in the third degree of burns can interfere with the effective diffusion of systemic antimicrobial agents in the wound where the number of microorganisms is usually very high. Enzymatic treatment of the wound increases the penetration of local antibiotics and stimulates wound healing [3, 14].

In addition, the alkaline keratinases of *B. pumilus* and *Staphylococcus auricularis* are known to inhibit the formation of biofilms by 86% and 50%, respectively, and also remove 0.4013 g and 0.3823 g of silver from 1 g of X-ray and photo film, respectively. Alkaline proteases of *Aspergillus versicolor* and *B. subtilis* ATCC 6633 also provide a good recovery of silver from X-ray films [14, 47].

New applications of keratinases are associated with the removal of ear sulfur, whitening of pearls, purification of contact lenses and the participation of microbial keratinases in the formation of silver nanoparticles [14, 47].

Several groups of researchers studied the potential of keratinases as agents of biocontrol. Keratinase produced by *S. maltophilia* R13 was effective against several fungal pathogens, including *Fusarium solani*, *F. oxysporum*, *Mucor* sp. and *A. niger*, which cause diseases of valuable plants and cultures. Keratinase synthesized by *Thermoactinomyces* also showed antimicrobial properties in relation to plant pathogens. Keratinase of *Bacillus* sp. 50-3, as already mentioned, is effective against agricultural pests such as nematodes [16, 17]. Also, this enzyme can be used against mosquitoes that are the carriers of many tropical diseases [14].

In recent years, the attention of scientists and physicians in different countries of the world is tied to such unusual animal proteins as prions. Prion is a nerve cell protein, necessary for its vital functions and normal functioning, which as a result of mutations becomes neurotoxic and capable of killing these cells, that is, it becomes an infectious unit. In this case, the PrP^{C} cell prion protein structurally converts into an incorrect fold form, known as PrP^{Sc}. The usual PrP^{C} protein is approximately 45% α-helix and only 3% β-sheets, and the abnormal PrP^{Ss} conformer is about 30% α-helix and 45% β-sheets. Such prion can cause a number of neurodegenerative diseases in humans and

animals with the formation of spongiform encephalopathy, which also belong to a group of slow infections and are characterized by damage to the central nervous system (CNS), muscle, lymphoid and other systems, and always lethal. The prevalence of prion diseases increases with each passing year. The prion can enter the environment in several ways: improper disposal after death, wrongly done disposal of biological materials, or sewage at slaughterhouses and in hospitals, as well as in case of waste processing of bone flour of infected animals. The basic methods used to eliminate prions, such as combustion, thermal and alkaline hydrolysis, are very rigid and energy-consuming and negatively affect medical instruments. Since the prion structure is highly similar to β-keratinous feathers, keratinases are capable of hydrolyzing it [14, 72], which provides an environmentally friendly and sustainable alternative for prion degradation. Various studies have been carried out on the use of microbial keratinases isolated from *Bacillus* sp., *Streptomyces* sp., *Nocardiopsis* sp. TOA-1 and thermophilic microorganisms such as *Thermoanaerobacter*, *Thermosipho* and *Thermococcus* sp. for the degradation of prions [3, 5, 14].

Keratinase produced by *B. licheniformis* PWD-1 can degrade the brain tissue of cattle infected with bovine spongiform encephalopathy and sheep scrapie in the presence of a detergent and at elevated temperatures (> 100 °C) [10]. Thirty two microbial strains were isolated on feather meal agar from primary effluent and farmyard wastes [45]. One of the isolates, a Gram positive bacterium, demonstrated significant keratinase activity (11.00 ± 0.71 U/ml). The isolate was identified by 16S rDNA and designated as Bacillus licheniformis N22. The growth conditions for optimum keratinase synthesis in a minimal growth medium (MGM) were found to be pH 8.5, 50 °C, 1.1 % (w/v) feather meal substrate and at incubation time of 32 h. The molecular weight of purified keratinase was ≈28 KDa as measured by SDS-PAGE and confirmed by MALDITOF-MS. Optimum keratinase activity was obtained at pH 8.5 and 50 °C. This keratinase fully degraded recalcitrant melanised feather in 48 h, and also digested ME7 scrapie prion at 65 °C in 2 h to levels of PrP^{Sc} undetectable by Western blot analysis. In a remarkable synergistic enzymatic preparation composed of keratinase and biosurfactant derived from Pseudomonas aeruginosa NCIMB 8626, ME7 scrapie prion was degraded to undetectable levels at 65 °C in 10 min. Interestingly biosurfactant alone showed no detectable activity on ME7 scrapie prion. Time-course degradation analysis showed progressive attenuation of PrPSc signal at 50 °C over time. Test of residual infectivity by standard cell culture assay showed that this enzymatic method completely destroyed standard sheep scrapie prion (SSBP/1) at 65 °C in 1 h. The mean survival time of mice challenged with enzyme digested inoculum significantly increased from 278 ± 9 days to 334 ± 42 days compared to those inoculated intraperitoneally with neat ME7 scrapie ($p = 0.008$ at 95 % confidence interval). Furthermore, 47 % of all the mice in enzyme-digested group lacked detectable levels of PrP^{Sc}. These results suggest a substantial reduction in the infectious titre or complete destruction of ME7 prion infectivity by the enzymatic preparation. Therefore, this mild enzymatic treatment method has potential applications for prion decontamination.

Currently, three commercial keratinase enzyme preparations are used to degrade infectious prion proteins: Versazyme® (BRI), Pure100 Keratinase ™ (Proteos Biotech), and Prionzyme ™ (Genencor International). Prionzyme ™ removes prions from medical and dental instruments with its effective enzymatic decontamination technology [72].

Thus, in recent years, interest in the study of keratinases has increased significantly. This, above all, is due to the annual growth of poultry farms and livestock production, as well as the continuous expansion of the use of these enzymes. The keratinase preparations today are quite expensive (such as the keratinase produced by Merck) and can not meet the growing needs of these enzymes. In Ukraine, only one keratinase preparation, ENZIM (ENZIM Group, Corporation, Ladyzhyn, Vinnytsia Region) is available, but there is no information on the basic physico-chemical characteristics of this enzyme. Therefore, the search for new producers of keratinases and study of their properties are an important area of scientific research, which has not only a fundamentally scientific aspect but also a significant ecological and biotechnological potential.

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КЕРАТИНОЛІТИЧНІ ЕНЗИМИ МІКРООРГАНІЗМІВ: ПРОДУЦЕНТИ, ФІЗИКО-ХІМІЧНІ ВЛАСТИВОСТІ. ВИКОРИСТАННЯ ДЛЯ БІОТЕХНОЛОГІЇ

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Метою огляду було проаналізувати сучасні уявлення про кератинази, групу протеолітичних ензимів, які каталізують розщеплення високостабільних фібринових протеїнів — кератинів. Представники різних таксономічних груп мікроорганізмів, включаючи гриби, актиноміцети і бактерії, є продуцентами кератиназ. В огляді наведено сучасну класифікацію кератиназ відповідно до бази даних MEROPS. Вона ґрунтується на схожості амінокислотних послідовностей, що також відображає еволюційні взаємодії між протеолітичними ензимами. База даних MEROPS об'єднує протеази в 62 клани і 264 родини. Дослідження фізико-хімічних властивостей кератиназ свідчать, що ензими активні в широкому діапазоні температур і значень pH, з оптимальною дією за нейтрального і лужного pH і 40–70 °C. Показано, що мікробні кератинази є переважно метало-, сериновими або метало-сериновими протеазами. Вони зазвичай позаклітинні, і їх синтез індукується кератиновмісними субстратами. В огляді обговорюється практичне застосування кератиназ. Ці ензими успішно використовують у біоконверсії кератинових відходів у корми для тварин і азотні добрива, а також у шкіряній, текстильній, косметичній, медичній та фармацевтичній промисловості. Кератинази також застосовують як пестициди у виробництві наночастинок, біопалива, біоплівок, що розкладаються, клеїв і фольги. Крім того, кератинази використовують у деградації пріонових протеїнів, які здатні спричинити низку нейродегенеративних захворювань людини і тварин з утворенням губчастої енцефалопатії.

Ключові слова: кератинази, продуценти, регуляція синтезу, фізико-хімічні властивості.

КЕРАТИНОЛИТИЧЕСКИЕ ЭНЗИМЫ МИКРООРГАНИЗМОВ: ПРОДУЦЕНТЫ, ФИЗИКО-ХИМИЧЕСКИЕ СВОЙСТВА. ПРИМЕНЕНИЕ ДЛЯ БИОТЕХНОЛОГИИ

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Целью обзора было проанализировать современные представления о кератиназах, группе протеолитических энзимов, катализирующих расщепление высокостабильных фибриновых протеинов — кератинов. Представители различных таксономических групп микроорганизмов, включая грибы, актиномицеты и бактерии, являются продуцентами кератиназ. В обзоре приведена современная классификация кератиназ в соответствии с базой данных MEROPS. Она основана на сходстве аминокислотных последовательностей, что также отражает эволюционные взаимодействия между протеолитическими энзимами. База данных MEROPS объединяет протеазы в 62 клана и 264 семейства. Исследования физико-химических свойств кератиназ показывают, что энзимы активны в широком диапазоне температур и значений pH, с оптимальным действием при нейтральном и щелочном pH и 40–70 °C. Показано, что микробные кератиназы являются преимущественно металло-, сериновыми или метало-сериновыми протеазами. Они обычно внеклеточные, и их синтез индуцируется кератиновыми субстратами. В обзоре обсуждается практическое применение кератиназ. Эти энзимы находят применение при биоконверсии кератиновых отходов в корма для животных, азотные удобрения, а также в кожевенной, текстильной, косметической, медицинской и фармацевтической промышленности. Кератиназы также применимы как пестициды в производстве наночастиц, биотоплива, биоразлагаемых пленок, клеев и фольги. Кроме того, кератиназы используются в деградации прионных протеинов, которые способны вызывать ряд нейродегенеративных заболеваний человека и животных с образованием губчатой энцефалопатии.

Ключевые слова: кератиназы, продуценты, регуляция синтеза, физико-химические свойства.

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