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The Tale of a Versatile Enzyme: Molecular Insights into Keratinase for Its Industrial Dissemination

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ABSTRACT

Keratinases are unique among proteolytic enzymes for their ability to degrade recalcitrant insoluble proteins, and they are of critical importance in keratin waste management. Over the past few decades, researchers have focused on discovering keratinase producers, as well as producing and characterizing keratinases. The application potential of keratinases has been investigated in the feed, fertilizer, leathering, detergent, cosmetic, and medical industries. However, the commercial availability of keratinases is still limited due to poor productivity and properties, such as thermostability, storage stability and resistance to organic reagents. Advances in molecular biotechnology have provided powerful tools for enhancing the production and functional properties of keratinase. This critical review systematically summarizes the application potential of keratinase and in particular certain newly discovered catalytic capabilities. Furthermore, we provide comprehensive insight into mechanistic and molecular aspects of keratinases including analysis of gene sequences and protein structures. In addition, development and current advances in protein engineering of keratinases are summarized and discussed, revealing that the engineering of protein domains such as signal peptides and pro-peptides has become an important strategy to increase production of keratinases. Finally, prospects for further development are also proposed, indicating that advanced protein engineering technologies will lead to improved and additional commercial keratinases for various industrial applications.

Keywords: keratinase; sequence; structure; expression; protein engineering

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1. Introduction

Keratinase is a unique enzyme that can specifically hydrolyze highly rigid keratin wastes such as wool, feathers, and hair. These major byproducts of agroindustry are produced in large quantities worldwide every year, but are difficult to dispose of due to their tight structure and high disulfide-bond content. The accumulation of such wastes has raised environmental concerns and resources squandering. The discovery and utilization of keratinase has effectively solved this problem, making it a research hotspot over the past few decades, with many studies focusing on keratinase identification, production, characterization, and application (Brandelli, 2008; Verma et al., 2017).

Keratinase can be produced by a wide range of microorganisms in nature, from prokaryotes to eukaryotes (Calin et al., 2017; Gegerekas et al., 2018; Tatineni et al., 2008). Most of these microorganisms are isolated from keratin-rich environments, such as decomposing feathers (Nagal & Jain, 2010), poultry wastes (Williams et al., 1990), and slaughterhouse wastes (Nangali & Brandelli, 2000a). The marine environment is also an important source of keratinase-producing strains. Herzog et al. discovered 50 unique bacterial species with keratin degradation ability from deteriorated feather samples on marine shorelines (Herzog et al., 2016). *Bacillus amyloliquefaciens* S13 was isolated from marine brown alga and found to produce two keratinases (Hariche et al., 2019). In recent years, researchers have found that animals also contain keratinase-producing strains. A novel *Bacillus subtilis* strain was isolated from the gut of a tarantula, and showed keratinase activity towards feathers, wool, weeds, and oil (Liu et al., 2017). Another study further demonstrated this by isolating keratinolytic bacteria from crocodile feces (Mamangkey et al., 2019). Moreover, a recent study reported a novel keratinase-producing bacterium from tofu liquid waste (Suharti et al., 2019). These new findings enhance our understanding of the broader and more pervasive presence of keratinases, which also imply the extensive substrate spectrum of keratinases and their important role in a variety of environments.

Biochemical properties vary widely among keratinases due to their different sources; these variations appear mainly in the enzyme structure, composition, stability, optimum temperature, and optimum pH value. Generally, reaction temperatures for keratinases are mild (40-60 °C), though keratinases secreted by strains isolated from near volcanoes and hot springs are usually thermophilic (Bouacem et al., 2016; Kuo et al., 2012). Similarly, most keratinases are neutral or alkaline enzymes with an optimal pH value between 7 and 10. However, a few saprophytic fungi on the surface of human skin produce acidic keratinases. For example, the optimal pH of keratinase from *Trichophyton mentagrophytes* is 5.5 (Tsuboi et al., 1989), and the optimal pH of keratinase from *Candida albicans* is 4.0 (Hattori et al., 1984), which may be related to the weak acidity of the human skin environment. Keratinases produced by microorganisms isolated from halophilic environments show the corresponding halophilic property (Arokiyaraj et al., 2012). This provides some guidance for screening keratinases with specific characteristics to meet application requirements. Environment and conditions are the main driving factors for the adaptive evolution of biological enzymes that confer specific properties.

This is demonstrated by citing a few reviews discussing the isolation of keratinase-producing microorganisms, and the production, characterization, and application of keratinases (Gupta et al., 2013a; Daroit & Brandelli, 2014; Gopinath et al., 2015; Adelere & Laeef, 2016; Ghaffar et al., 2018; Srivastava et al., 2020) (Fig. 1). To date, however, a limited number of keratinases have been used in commercial applications. This is because the inefficient productivity and poor catalytic properties of many keratinases for use in industrial applications. On the other hand, with the development of advanced molecular biotechnology, heterologous gene expression and protein engineering have evolved to be powerful means to efficiently acquire enzymes with specific properties. This review systematically summarizes the research and development of keratinases based on modern molecular biotechnology, provides comprehensive insight into keratinase genes and structures, and discusses heterologous expression and protein engineering modifications of keratinases. Finally,

we propose prospects in the forthcoming development of keratinases.

2. Catalytic performance

Keratinases have been hailed as critical enzymes because of their unique ability to attack highly rigid and insoluble keratin substrates, creating numerous applications in various agro-industrial and biotechnological sectors, as well as contributing to environmental protection and sustainable biomass development. During the past few decades, we have witnessed enormous progress in exploiting microbial keratinases for various potential applications (Onifade et al., 1998; Gupta & Ramnani, 2006; Brandelli et al., 2010) (Fig. 2).

2.1 From keratin wastes to valuable products

The most important application of keratinases is the bioprocessing of keratin-rich wastes, which are generated and accumulated as byproducts of agro-industrial processing. Such wastes are quality resources with high protein content, a variety of essential amino acids, major and trace elements, vitamins, and growth factors. Keratinases realize the conversion from these wastes to value-added products. For example, keratinase from *Geobacillus stearothermophilus* has been used to produce high-value small peptides from wool wastes. These peptides are potential major additives in animal feed, fertilizers, plant biostimulants, and nutritional supplements (da Silva, 2018b; Gegeckas et al., 2015). Similarly, chicken feather wastes have been degraded by keratinase-producing strains, and large quantities of antioxidant substances, amino acids, and soluble peptides were produced (Peng et al., 2019). Amino acid composition analysis of feather keratin hydrolysates revealed that glutamic acid and proline were the dominant products, as well as serine and glycine, and significant amounts of essential and branched-chain amino acids were also observed (Ciurko et al., 2019). Although porcine bristles demonstrate high mechanical strength, they were also hydrolyzed by keratinase from *Amycolatopsis keratiniphila*, with soluble proteins, small peptides, and free amino acids recovered (Falco et al., 2019).

So far, most keratinase hydrolysis products are small peptides and amino acids.

However, keratins with high molecular weights extracted from hair, wool, and feathers have been reported to be excellent biomaterials that are biocompatible, bioactive, and biodegradable (Hill et al., 2010). Thus, keratin-derived biomaterials have widespread applications in tissue engineering, drug delivery, and surgical dressing (Patrucco et al., 2016). Studies on extracting keratins using chemical reagents and physical treatments have been widely published (Shavandi et al., 2017), but there are few reports on keratin extraction by keratinases (Su et al., 2020; Ye et al., 2020). This is because of the difficulties in maintaining the intact structure and protein skeleton of desired end-products through the degradation process. We believe that under moderate conditions, keratins can be extracted by the controllable biodegradation of keratinases, and keratinases should be developed as a green alternative to replace toxic and hazardous chemicals. The development of keratin biomaterials based on keratinase extraction should be a critical research focus.

In addition to protein products, including peptides and amino acids, studies have demonstrated the excellent performance of keratinases in the extraction of non-protein products from various keratinized substrates. Glucocorticoids mediate the biological stress response, and their recurrent activation can suppress reproduction, growth, and immunity in animals. A method of glucocorticoid extraction from chicken feathers using keratinase was developed to facilitate conservation breeding and livestock production (Alba et al., 2019). In addition, Pernicova and her coworkers connected keratinase biodegradation of feather wastes with production of medium-chain length polyhydroxyalkanoate (Pernicova et al., 2019). A recent study reported that the hydrolysates of hair wastes generated by a keratinase could be utilized by a *Saccharomyces cerevisiae* strain to produce vitamin B-complex including vitamins B1, B2, and B12 (Hassan et al., 2020). Thus, keratinase facilitates the bioprocessing of keratin solid wastes and the production of both protein and non-protein value-added products, with applications in agriculture, poultry, fishing, cosmetics, and medicine.

2.2 Dehairing

Dehairing is another important application of keratinases, especially in leather

industry. Keratinases can replace the sodium sulfide and lime used in traditional dehairing processes, achieving safe and effective hair removal. Animal skin is mainly composed of structural proteins such as collagen and elastin. In the process of leather making, excessive hydrolysis of collagen should be avoided to maintain the quality of leather including grain state, softness, elasticity and fullness. Keratinase is a broad-spectrum protease that can also be active on various soluble proteins such as casein, gelatin and collagen. These activities are not specific and mainly depend on the ability of keratinase to bind with the substrate, which is associated with the structural conformation, steric resistance, hydrophobic force and electrostatic environment of the substrate binding region of keratinase (Perona & Craik, 1995). Therefore, the subtle structural differences in substrate binding region of keratinase and type of amino acid residues at the binding site might lead to different substrate specificities. For leather dehairing application, the keratinase without collagen activity is of great significance. Zhang et al. identified two keratinases with high keratinase activity but low/no collagenase activity. Such keratinases could obviously remove the epidermis and hair follicles from the skin while well preserving the inherent collagenous corium structure (Zhang et al., 2016b; Zhang et al., 2016c). Kalaikumari et al. verified that keratinase could achieve 100% dehairing of sheepskin with obtaining a high-quality leather crust (Kalaikumari et al., 2019). They also assessed the wastewater effluent and confirmed a significant reduction in environmental contaminants, indicating that keratinase dehairing is an eco-friendly alternative to the detrimental conventional process. Tian et al. further characterized the pelt after keratinase dehairing and revealed a better smooth and white appearance, as well as a more opened and regular collagen fiber structure with the epidermis completely removed (Tian et al., 2019a). Thus, we conclude that keratinase is a promising candidate for the dehairing process in the leather industry.

2.3 Effects on skin and tissues

Many findings indicate that keratinases act upon human skin as well as other keratinous tissues and filaments, suggesting potential applications in cosmetics and

pharmaceuticals. Keratinases have been investigated for inclusion in hair removal cream due to their mild dehairing properties (Sanghvi et al., 2016). They are also added to cosmetic formulas for skin care, with whitening, freckle-removal (Yang et al., 2012), anti-wrinkle, and anti-skin aging effects (Yeo et al., 2018). Studies have demonstrated the remarkable curative effects of keratinases in the treatment of hyperkeratosis, such as in corns, calluses, and psoriasis (Gupta et al., 2017). Similarly, keratinases are effective in treating acne and pimples, common skin problems caused by excessive keratin accumulation in sebaceous glands (Spyros et al., 2003). Keratinases may be used as an auxiliary agent to promote the effective adsorption of cosmetics and medicines (Mohorcic et al., 2007). Keratinase KerN has been reported to enhance the penetration of drugs through the nail, thus increasing the bioavailability of these drugs (Tiwary & Gupta, 2010b). Some commercial keratinase-based enzymes have been used to treat nail diseases: FixaFungus™, kernail-Soft PB, and Pure100 Keratinase (Sharma & Devi, 2018).

2.4 Removal of protein stains/contaminants

With increasing emphasis placed on environmental protection, the use of biocatalysts in detergents has gained wide attention. Keratinase have shown their application values in this market for they not only effectively remove a variety of soluble and insoluble protein stains, such as blood, milk, egg yolk, and egg white (Gong et al., 2015; Zhang et al., 2016a), but also exhibit excellent ability to remove stubborn keratinous contaminants produced by human skin such as scurf that often attach to shirt collars and cuffs (Paul et al., 2014). This kind of dirt cannot be easily hydrolyzed by common proteases. On the other hand, many alkaline keratinases have been reported to remain active and stable in washing conditions where surfactants, oxidants, detergents and solvents are present (Su et al., 2017). Therefore, they are considered as strong “green additives” in detergents and their utilization reduces the amount of surfactant and hazardous chemical reagents used, making a significant contribution to the environmental safety of detergents (Manivasagan et al., 2014). Another application of keratinases in detergents is to clean drains and pipes clogged

with hair and other keratin wastes. The commercial product BioGuard Plus is a mix of enzymes containing keratinase used to clean water tanks and drainage pipes (Gupta et al., 2013a).

2.5 Extended catalytic performance

2.5.1 Hydrolysis of amyloids

In addition to specific hydrolysis of keratin substrates, keratinases have shown other catalytic abilities. Prions are amyloids that cause mad cow disease and Kuru. Their cross-sheet abnormal structure makes prions insoluble and resistant to common proteases (Prusiner et al., 1998). Studies have shown that keratinase efficiently degrades these prion proteins (Langeveld et al., 2003; Müller-Hellwig et al., 2006). Thus, keratinase can be adopted as a “cleaning agent” for surgical instruments suspected of prion contamination. Furthermore, research should be conducted regarding keratinase utilization in the treatment of diseases caused by prion infection.

A β fibrils have similar cross-pleated sheet structures to prions that contribute to Alzheimer’s disease (AD). Recently, a study showed that keratinases are novel drug molecules that help combat AD by degrading β -amyloid fibrils (Ningthoujam et al., 2019). Another study drew a similar conclusion, that keratinase-guided peptides could inhibit the aggregation of amyloid- β , thus paving the way for developing effective therapeutics for AD and other amyloid diseases (Rajput et al., 2019).

2.5.2 Biosynthesis of nanoparticles

Keratinases are proved to exhibit the ability to biosynthesize metal nanoparticles, especially silver and gold nanoparticles (AgNPs/GNPs). These nanoparticles are important candidates in oncotherapy, X-ray imaging, targeted-drug-delivery, and sensing applications due to their biocompatibility, chemical inertness, and antimicrobial activity (Levy et al., 2010; Rai et al., 2014). Lateef et al. reported green synthesis of AgNPs by taking advantage of the bio-reductivity of keratinase secreted by *Bacillus safensis* LAU 13, and the AgNPs produced showed excellent antimicrobial properties (Lateef et al., 2015). Subsequently, keratinase from *Stenotrophomonas maltophilia* was reported to be responsible for the eco-friendly

synthesis of AgNPs by reducing silver ions (Jang et al., 2018). Tao et al. further verified this ability of keratinase by using the keratinase expressed by *B. subtilis* WB600 as a reducing agent for the biosynthesis of AgNPs (Tao et al., 2018). Similarly, keratinase was applied to synthesize GNPs, and the products were characterized as nontoxic (Gupta et al., 2015). Compared with physicochemical synthetic method, biological process is considered more economical and environmentally friendly, and the products are more stable (Hulkoti & Taranath, 2014). In these studies, keratinase was used as reducing agent for the bio-synthesis of Ag/Au nanoparticles. There are many deduced mechanisms for this reduction: keratinase has been considered to have disulfide reduction activity; also, some studies indicated that the presence of free and exposed S-H groups in cysteine was acting as reductant, as previously described for the enzymatic preparation of silver nanoparticles by *laccase* (Duran et al., 2014) and gold nanoparticles by *amylase* (Rangnekar et al., 2007). In the case of lysozyme-based synthesis of silver nanoparticles, hydroxyl groups in tyrosine residues and amine groups in tryptophan residues were found to be responsible for this production (Kumar et al., 2012).

2.5.3 Decolorization of molasses wastewater

Current studies have revealed that many enzymes can catalyze various non-specific reactions. For instance, protease and other hydrolases were reported to catalyze addition reaction and hydration reaction of olefins (Svedendahl et al., 2009). Previous researches suggest that the active sites of non-specific catalytic in hydrolases are still located in the original active site of enzymes, along with oxyanion hole activating functional groups with unsaturated bonds (Cai et al., 2006; Xu et al., 2007). According to this, Zhang et al. reported a keratinase-based decolorization of melanoidins from molasses wastewater whose chromogenic group is a conjugated olefins system (Zhang et al., 2019). They confirmed that keratinase could destroy the conjugated system of melanoidins by catalyzing the addition reaction of unsaturated C=C bond with H₂O. They also demonstrated that the non-specific catalysis of keratinase is associated with its active site and oxyanion hole, which can polarize and

activate functional groups with unsaturated bonds. These results suggest the ability of keratinase to catalyze the addition and hydration reactions of unsaturated bonds and indicate its application potential in the treatment of colorant wastewater.

2.6 Determination and definition of keratinase activity

The main principle for keratinase activity determination is to assay the content of peptides and amino acids released after hydrolysis or to detect degradation degree of keratin substrate. Different methods for determining keratinase activity have been developed based on the choice of different substrates. At present, the most used substrates in keratinase activity determination are azo keratin (Lin et al., 2009a; Huang et al., 2017), azure keratin (Nahar et al., 2016; Pawar et al., 2018), soluble keratin (Fang et al., 2014; Su et al., 2017; Wang et al., 2019), and feather powder (Jaouadi et al., 2013; Gupta et al., 2017; Jin et al., 2019; Yong et al., 2020). It must be pointed out that considering the substrate differences in solubility and composition, as well as substrate preference of keratinase, the activity determined by different substrates may differ greatly. Hu (Hu et al., 2013b) detected the activity of a recombinant keratinase using azure keratin and chicken feather as substrate, respectively. It turned out that keratinase activity with chicken feather as substrate was significantly higher (nearly 2-fold) than that with azure keratin as substrate. Meanwhile, even with the same substrate, detection conditions are usually modified according to the characteristics of the specific keratinase, such as pH, temperature and reaction time, leading to different definition of keratinase activity. For example, Sangali (Sangali and Brandelli, 2000b) assayed a keratinase activity with azo keratin as substrate at pH 8.0, 50 °C for 15 min; thus, the keratinase activity was defined as the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm for 15 min at 50 °C. Another case (Huang et al., 2017) also assayed keratinase activity using azo keratin substrate, but at the condition of 37 °C for 30 min, for which the keratinase activity was defined as an increase in A_{450} of 0.01 at 37 °C for 30 min. This gives the indication that a keratinase activity should be understood along with its substrate and definition.

The K:C value (keratinolytic activity: caseinolytic activity) is put forth to judge the potentiality of an enzyme as keratinase, which is also taken as a standard parameter to distinguish keratinases from other conventional proteases (Evans et al., 2000). A hydrolase with a K:C > 0.5 is considered as a potential keratinase, which is well supported by the high K:C value of the well-known keratinase from *Bacillus pumilus* KS12 (1.33) and *Bacillus subtilis* MTCC (1.42) (Gupta et al., 2013b). On the contrary, non-keratinolytic proteases exhibit low K:C value such as trypsin (0.08), papain (0.02) and proteinase-K (0.28). For determining K:C ratio, a standard method is specified with feather keratin as the substrate for keratinolytic activity assay and casein for caseinolytic activity assay.

2.7 Hydrolytic specificity

Keratinase should not be merely considered as an enzyme capable of hydrolyzing keratin substrates, but a specific protease that can adhere to and hydrolyze insoluble proteins (Yoshioka et al., 2007; Ningthoujam et al., 2019). Their ability to specifically cleave the hydrophobic and aromatic amino acids has been demonstrated by using synthetic *p*-nitroanilide derived peptides as substrates (Brandelli et al., 2010). Studies revealed that keratinases were strongly active on Suc-Ala-Ala-Pro-Phe-*p*Na by cleaving at P1 site (Phe) (Gradisar et al., 2005; Mitsuiki et al., 2004; Moallaei et al., 2006). This cleavage specificity was further verified on CBz-Phe-*p*Na and CBz-Phe-*o*Np substrate, showing the preferable cleavage of the hydrophobic and aromatic amino acid residue Phe at the P1 position (Bockle et al., 1995; Thys & Brandelli, 2006).

Hydrolytic specificity of keratinase has also been studied on insulin B chain (F-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A) (Gupta et al., 2013b). Keratinases from different microbial sources exhibited their unique cleaving preferences. Keratinase from *Thermoanaerobacter* sp. has four cleavage sites located between Cys⁷-Gly⁸, Leu¹¹-Val¹², Leu¹⁵-Tyr¹⁶, and Phe²⁴-Phe²⁵ (Kublanov et al., 2009); while totally different, keratinase from *Bacillus pumilus* KS12 has three cleavage sites located between Val²-Asn³, Leu⁶-Cys⁷, and His¹⁰-Leu¹¹ (Rajput et al.,

2010). The results further confirmed the cleavage preference of keratinase to hydrophobic amino acid residues such as phenylalanine, leucine, valine and tyrosine, although the preference of different keratinases was not the same.

In summary, the specific catalytic performance of keratinases towards insoluble and rigid proteins, such as but not limited to keratins, as well as the widespread presence of these proteins in nature and the human body promote the extensive application of keratinases in multiple fields. Utilization of keratinases is both economically and environmentally important.

3. Mechanism of keratin degradation

Degradation of keratin substrates by keratinases is a complex process, and the mechanism has not been fully clarified yet, which is the most controversial issue for keratinase at present. It is widely recognized that the degradation process involves reduction of disulfide bonds and hydrolysis of proteins, though researchers have expressed different views on the source of the reducing power (Fig. 3). Ramnani et al. have claimed that the efficient degradation of keratin substrates necessitates the assistance of sulfitolysis (Ramnani et al., 2005). The sulfitolysis is the reaction of cleavage of disulfide bonds by sulfite ions including sulfite (SO_3^{2-}), bisulfite (HSO_3^-), and disulfide ($\text{S}_2\text{O}_5^{2-}$), which results in the formation of a thiol and an S-sulphonate anion (bunte salts). Sulfite ions (SO_3^{2-}) react with cystine more rapidly than bisulfites (HSO_3^-), and higher rate of sulfitolysis are achieved by increasing the pH (Vineis et al., 2019). Cedrola et al. also speculated that sulfide likely participates in the breakdown of disulfide bonds in feather keratin because they detected the presence of sulfide in extracellular medium, and found that external addition of sulfide further increased feather degradation (Cedrola et al., 2012). In addition to sulfite-assisted hydrolysis, reductase-assisted hydrolysis has also been suggested; the degradation process is considered as the cooperative action of keratinase and reductase (Lange et al., 2016). Yamamura et al. discovered two types of extracellular proteins from a newly isolated bacterium: a serine protease and a disulfide reductase. They found that mixing the two enzymes significantly increased keratinolytic activity, while neither of

the enzymes showed independent activity (Yamamura et al., 2002). Contrary to the aforementioned synergistic actions of sulfitolysis or reductase, some studies have shown that keratinase is a multi-functional enzyme with both reductase and hydrolase activity (da Silva, 2018c). Prakash et al. purified two keratinases from *Bacillus halodurans* PPKS-2 and demonstrated that both showed dual hydrolase-reductase activity (Prakash et al., 2010). Additionally, one separated and purified keratinase was identified to possess capabilities for hide depilation and feather disintegration, suggesting that degradation of keratin substrate by keratinase is independent of the presence of sulfite or reductases (Pillai et al., 2011).

In summary, disulfide bond reduction and polypeptide hydrolysis are both essential for keratin substrates degradation, and current research indicates that keratinase is a multifunctional enzyme with both hydrolyzing and reducing activities. Except for keratin degrading, its reducibility has also been demonstrated by being used as reductant in the biosynthesis of metal nanoparticles. Multifunctional enzymes (MFEs) have been identified to play multiple physiological roles, which are considered as the results of natural evolution of organisms (Zou & Xiao, 2016). MFEs may simultaneously belong to two or more functional classes with single or multiple catalytic domains. The hydrolytic active center of keratinases has been clarified while their reducing activities may relate to this center or there may be specialized fragments in keratinase that are responsible for the reducibility. Except for the cysteine groups we have discussed, some special sequences and structures should be focused on, such as a specific region containing the CXXC structure of a thioredoxin. Therefore, further researches are needed to explore the reducibility of keratinases, and it enlightens us to pay attention on the characteristic sequences and structural domains in keratinases. The clarification of the reducibility of keratinase will help to unravel the degradation mechanism of keratinase and further guide researches and applications.

4. Keratinase mining and heterologous expression

4.1 Gene mining and sequence analysis

4.1.1 Gene mining

Exploring of novel and more powerful keratinases always remains significant in scientific and industrial research. With the development of molecular biology, genomics, bioinformatics and especially advanced DNA technologies, gene mining is considered as the important approach for exploring new enzymes (Gong et al., 2013). The natural environment is a vast reservoir of various microorganisms, functional enzymes, and encoding genes (Rajendhran & Gunasekaran, 2008). Conventionally, keratinase-producing strains are first screened and isolated using a selective medium with feathers or wool as the sole source of carbon and nitrogen. Then, keratinase genes are cloned from genomic DNA with a pair of degenerate primers designed based on the conserved sequence (Fig. 4A). For example, the genes for keratinase A (*kerA*), keratinase D (*kerD*) and F (*kerF*), and keratinase K (*kerK*) have been successfully identified and cloned from *P. ticheniformis* (Hu et al., 2013), *S. maltophilia* (Fang et al., 2014), and *E. amylovorifaciens* (Yang et al., 2016), respectively. More keratinase-producing microorganisms are being identified and gene mining based on strain isolation is considered feasible and effective; however, it is estimated that more than 90% of microorganisms in nature cannot be cultivated by traditional culture techniques. Therefore, metagenomic screening is a powerful tool for providing more genetic diversity and options (Handelsman et al., 1998; Xing et al., 2012).

Metagenomic mining is based on the construction of a metagenomic library of environmental samples directly without culturing, thus bypassing the culture barrier and expanding the utilization space of microbial resources (Ufarte et al., 2015) (Fig. 4B). Keratinase genes are then identified by functional screening of the library. A novel keratinase gene was mined from the metagenome of a soil sample by constructing a fosmid library, and positive clones were screened by the transparent zones in combination with activity assays (Su et al., 2017). Metagenomics has proven to be an effective strategy to mine novel genes by many reports of its applications in screening novel cellulase (Ilmberger & Streit, 2017), hydrogenase (Adam & Perner,

2017), and other enzymes (Schultz-Johansen et al., 2018).

With the rapid development of gene sequencing technology, a large number of microbial genomes have been sequenced, however, a great majority of gene sequences from these genomes are uncharacterized for their definite biological functions. Keratinase gene mining can be achieved by using the conserved sequences of keratinase as gene probes for sequence alignment in these databases to screen the coding information of homologous enzymes. The coding sequences can be chemically synthesized or amplified by PCR technique, and then heterologously expressed to obtain new keratinases with better catalytic performance via functional screening (Fig. 4C). At present, gene databases such as NCBI (<https://www.ncbi.nlm.nih.gov/>), GOLD (<https://gold.jgi.doe.gov/index>), EMBL (<https://www.ebi.ac.uk/>) and UniProt (<https://www.uniprot.org/database/DB-0028>) are open online for researchers, which offered an unprecedented chance for exploring interesting keratinases.

4.1.2 Sequence analysis

A number of keratinase genes have been successfully cloned and sequenced so far, and these are available in the NCBI library (Table 1). BLAST analysis reveals that most of the reported keratinases belong to the peptidase S8 family, members of which contain a peptidase_S8 region. In addition, the N-terminal sequence always has a great influence on the biological function of protein and can be used for identifying proteins (Manabe et al., 1985). For keratinase, its N-terminal sequence analysis reveals a high homology (>98%) with subtilisins. For example, the N-terminal sequence of *kerA* from *B. licheniformis* PWD1 is identical to that of subtilisin Carlsberg (AQTVPYGIPLIKADK) (Gupta & Ramnani, 2006). A novel keratinase from *B. subtilis* S14 possesses an N-terminal sequence identical to that of subtilisin E (AQSVPYGISQIKAPA) (Macedo et al., 2005). An oxyanion hole region (GVVVVAAAGN) is also found to maintain in high level of conservation (Gupta et al., 2013b). Keratinases are largely classified as serine or metallo proteases according to their catalytic type (Brandelli, 2008). Most keratinases are serine-type proteases with a conserved active center consist of Asp/His/Ser catalytic triad in which serine

acts as a nucleophile, aspartate as an electrophile, and histidine as a base (Jaouadi et al., 2013). These enzymes are strongly inhibited by phenylmethylsulfonyl fluoride (PMSF) and benzamidine. Metallo keratinases are reported to contain metal ions (Ca^{2+} , Zn^{2+}) in active center and they are inhibited by ethylene diamine tetraacetic acid (EDTA) and phenanthroline (Riffel et al., 2003). Keratinase from *Streptomyces* sp. was inhibited by both PMSF and EDTA, thus identifying as a serine metalloprotease (Tatineni et al., 2008). Sequence alignments reveal that keratinase genes derived from *B. licheniformis* maintain a high sequence homology of 99.38%. Similarly, keratinase genes from *B. pumilus*, as well as *P. tequilensis*, *B. circulans*, and *B. brevis*, also exhibited high homology of 97.21%. Keratinase genes *kerC*, *kerK*, and *kerBPN* share 93.19% sequence homology. However, some keratinases show significant differences in sequence, indicating gene diversity and variability.

4.2 Heterologous expression advancements

Industrial production of keratinase by natural producers has been prevented because wild types generally cannot meet industrial requirements, and some strains are pathogenic. As an increasing number of keratinase genes have been identified, heterologous expression of keratinases in engineered expression systems has attracted considerable attention. These expression systems are characterized by high productivity, operability, and security. Among these, *Escherichia coli*, *Bacillus*, and *Pichia* yeast are the most commonly used.

4.2.1 *Escherichia coli* expression system

E. coli is usually the system chosen to overexpress heterologous enzymes because of its rapid growth and simple biosystem (Zelena et al., 2014). As a host, it has successfully expressed many keratinase genes from different sources (Table 2). Keratinase genes from *B. licheniformis* S90 (*kerA*), *B. licheniformis* ER-15 (*kerBL*), and *B. licheniformis* MZK-05 (*kerA*) were cloned into *E. coli* HB101 and *E. coli* BL21 (Hu et al., 2013; Tiwary & Gupta, 2010a). Fang et al. separated two keratinase genes from *S. maltophilia* BBE11-1, *kerSMD* and *kerSMF*, and successfully expressed them in *E. coli* BL21 (DE3) (Fang et al., 2014). Two keratinase genes from *P.*

aeruginosa, a pathogenic bacterium, were separately expressed in *E. coli* AD494(DE3) pLysS and *E. coli* HB101 (Lin et al., 2009a; Sharma & Gupta, 2010). Moreover, the *keratinase* gene of *Bacillus* sp. JM7 derived from a deep-sea organism was cloned into *E. coli* BL21(DE3) for keratinase production (Jin et al., 2019). Despite the relatively clear genetic background, as well as traits for fast growth, high cell density, and easy operation, the *E. coli* expression system still has some problems, such as the formation of inactive inclusion bodies and low secretory efficiency. Keratinase retention within host cells can cause cytotoxicity and incorrect protein folding, leading to cell lysis and inactive precursors. Therefore, effective secretion is a key consideration in improving the productivity and activity of keratinases.

4.2.2 *Bacillus* expression system

Bacillus is a preferable host for gene secretory expression due to its excellent protein secretion ability (Fu et al., 2007). In this system, proteins tend to be secreted into the extracellular culture, promoting generation and accumulation of desired products. This also provides convenient downstream purification processing. *Bacillus subtilis* is generally regarded as a food-grade safe strain and is widely used for large-scale protein production in industry. *B. licheniformis* PWD-1 keratinase gene (*kerA*) was first expressed in *B. subtilis* DB104 by Lin et al. in 1997 (Lin et al., 1997). Afterwards, ten keratinase genes were successively expressed and secreted in the *B. subtilis* system (Table 2). However, expression by *B. subtilis* also presents some problems: redundant endogenous proteases are a limiting factor that may degrade the expressed protease, thus reducing the yield. *B. megaterium* has been discovered to be an excellent host for foreign protein production without endogenous protease (Rygus & Hillen, 1991). Radha et al. achieved stable expression of the *B. licheniformis* MKU3 keratinase gene in *B. megaterium* MS941 and *B. megaterium* ATCC 14945 (Radha & Gunasekaran, 2008). The instability of plasmids is another major disadvantage of the *Bacillus* expression system. For keratinase genes originating from eukaryotes, a eukaryotic expression host can be a more suitable candidate for efficient heterologous expression.

4.2.3 Yeast expression system

Yeast expression, especially using *Pichia pastoris*, is an attractive exogenous protein expression system developed in the early 1980s (Yang & Zhang, 2018). It shows the capabilities of post-translational modification for exogenous proteins, such as glycosylation and protein phosphorylation, that are not found in prokaryotic expression systems. To date, a variety of keratinases have been successfully expressed in this system (Table 4). For example, the keratinase genes from *A. fumigatus* and *P. aeruginosa* were expressed in *P. pastoris* SMD 1165 and *P. pastoris* SMD1168H (Noronha et al., 2002; Lin et al., 2009c). It is broadly recognized that expression level in the *P. pastoris* system strongly depends on codon usage bias (Boettner et al., 2007). Hu et al. achieved expression of *kerA* from *B. licheniformis* S90 in *P. pastoris* and improved keratinase production via codon optimization strategies by twelve-fold compared to expression in the *E. coli* system (Hu et al., 2013b).

4.2.4 Other expression systems

The parent cell can also be considered as an expression host for the over-expression of keratinase, when it is a safe and operable strain. In this case, keratinase productivity can be promoted by combining constitutive expression and plasmid expression. Yang et al. transformed recombinant plasmid containing the keratinase gene *kerK* into its parent host *B. amyloliquefaciens* K11, achieving 6-fold increased keratinolytic activity (Yang et al., 2016). Moreover, it has been reported that insect cells could be the host for expressing keratinase. Huang et al. reported the expression of the *B. licheniformis* PWD-1 keratinase gene *kerA* in insect *Spodoptera frugiperda* (Sf9) cells by virus infection (Huang et al., 2017). The findings demonstrate that, in addition to the heavily used expression systems mentioned above, there are many potential hosts to be explored and constructed for effective heterologous expression of keratinase.

5. Protein engineering strategies for keratinase

Keratinase genes from multiple sources have been successfully cloned and heterologous expressed, taking advantage of molecular biotechniques, but the product

is still not satisfactory. Keratinases are required to be more efficient, high-producing, and thermostable in industrial applications and commercial markets than they currently are (Li et al., 2013b). Protein engineering has witnessed stunning growth in recent decades, and techniques are rapidly maturing, providing tools to alter protein structure, leading to new characteristics and higher productivity (Bornscheuer et al., 2012; Liu et al., 2019). This technology has been used on various keratinases to promote production, activity, and thermostability via different strategies (Table 5).

5.1 Structure analysis

Protein engineering is conducted based on analysis of protein structure and function. The crystal structure of keratinase MtaKer from *Meiothermus taiwanensis* WR-220 (PDB: 5WSL) was reported by Wu et al. (Fig. 5A), indicating that MtaKer consists of three parts: signal peptide (pre), N-terminal pro-peptide (pro), and mature protease. MtaKer possessed the highly conserved catalytic triad residues Asp39, His72, and Ser224 (Wu et al., 2017). Two calcium ion-binding sites were also found in the structure of mature keratinase, playing essential roles for correct folding and structure stabilization. In addition, the 1.7 Å resolution crystal structure of a keratinase from *Fervidobacterium pennivorans* was also analyzed by Kim et al (Kim et al., 2004) (Fig. 5B). This revealed that the protease was composed of four domains: a catalytic domain, a pro-peptide domain, and two β -sandwich domains. The architecture of the catalytic domain closely resembles that of a subtilisin, with the catalytic triad of Ser189, His208, and Asp170 corresponding to Ser221, His64, and Asp32 in subtilisin. The calcium-binding site within the catalytic domain matched exactly with subtilisin E. It was also found that the pro-peptide domain fits into the curved surface of the binding pocket exactly, and the activation of the mature part requires cutting of the pro-peptide (Fig. 5C). This observation helps to clarify the keratinase maturation process (Fig. 6). Keratinase is first synthesized as an inactive processor with a pro-peptide domain to prevent degradation of intracellular functional proteins (Birkedal-Hansen, 1995). The pro-peptide domain is essential for guiding the correct folding of the mature protease as an intramolecular chaperone (Jain et al.,

1998). However, it is not required for enzymatic function of the mature protease, and even acts as an inhibitor to block binding with substrates (Zhu et al., 1989). The pro-peptide domain is cut off and degraded after completion of the folding process, and the active mature keratinase is released. In summary, the crystal structures of keratinases suggest functional relationships between the non-catalytic domains (pre-, pro-) and the catalytic center, providing a blueprint for potential protein engineering strategies.

Structural information further confirms that most of the reported keratinases are subtilisin-like protease, consistent with sequence homology analysis. Sporadically, some are also structurally categorized as chymotrypsin-like protease, such as keratinases from *S. albidoflavus* (Bressollier et al., 1999) and *Nocardiosis* sp. TOA-1 (Mitsuiki et al., 2004). *B. licheniformis* K 508 secreted an unusual trypsin-like thiol protease with keratinolytic activity (Pozs et al., 2001). Accordingly, for keratinases whose crystal structures have not been resolved, structural models can be assembled based on the crystal structures of subtilisins with high homology. The three-dimensional structure of keratinase from *B. licheniformis* BBE11-1 was modelled using the crystal structure of subtilisin Carlsberg (PDB: 3unxA) as template (Liu et al., 2013b). Homology models of keratinases KerSMD and its mutants (Fig. 7A and 7B) were constructed with *Vibrio* extracellular metalloprotease vEPCter 100 (PDB: 2LUW) as a template for modeling the C-terminal domain, and per BprV protease (PDB: 3TI9) for modeling the S1 pocket (Fang et al., 2016b). Moreover, the catalytic domain of KerSMD (Fig. 7C and 7D) was predicted based on the crystal structures of subtilisin-like proteases (PDB: 3LPA, 3LPC, 3LPD, 3TI9, 3TI7, 1DBI, 1THM, 3AFG), and the PPC domain based on kexin (PDB: 1OT5) (Fang et al., 2015). Homology structure modeling can be an effective and easy method to analyze the effects of mutations; it also provides a basis for protein engineering modifications.

5.2 Signal peptide optimization

The rapid expression and production of keratinase might be blocked by inefficient secretion; the intracellular accumulation of keratinase may destroy

important metabolism processes and structures of the host cell by acting on metabolism-related enzymes and cell membrane proteins, thus leading to cell lysis and death (Fang et al., 2019). Therefore, efficient secretion is of vital importance for keratinase production. The first and most important factor to be considered to enhance the secretory production of keratinase in heterologous hosts is the screening of optimal signal peptides, especially for the *Bacillus* expression system. Proteomics studies have shown that there are four secretion pathways in *B. subtilis*, and approximately 300 proteins secreted extracellularly (Tjalsma et al., 2004). The N-terminal sequence of these secreted proteins carry a specific secretion signal known as signal peptides. Signal peptides are short peptide chains that guide the transfer of newly synthesized proteins to the secretory pathway. During secretion, signal peptides play at least three functions, including avoidance of nascent chains misfolding inside the cells, recognition and submission to the secretion machinery, and as a topological determinant for preproteins in the membrane (Fu et al., 2007). Tian et al. screened five Sec-type signal peptides (SP_{Ync M}, SP_{Ync A}, SP_{Npr E}, SP_{Vpr}, SP_{Yvg O}) and four Tat-type signal peptides (SP_{Ywb N}, SP_{Lip A}, SP_{Amy X}, and SP_{Wap A}) to improve extracellular expression of keratinase. In this screen, keratinase with SP_{Lip A} showed 1.95-fold higher extracellular activity than wild-type (Tian et al., 2019b). Fu et al. constructed a library containing 173 different Sec-type signal peptides to study the influence of signal peptides on secretion efficiency and revealed that there is hardly any universal optimal signal peptide for all heterologous proteins; the optimum signal peptide for one recombinant protein was not always the best choice for another target protein (Fu et al., 2018). Therefore, the specific signal peptide optimal for each target protein should be screened and identified to facilitate secretory expression. They further explored the potential properties of the signal peptides that contribute to high secretion efficiency, and revealed some common properties of the 15 best-performing signal peptides. First, a net positively charged N-domain and a frequent occurrence of lysine in this region were observed. This helps target the signal peptides into the inner membrane by electrostatic interactions between the negatively charged lipid head

groups and the lipid layer, which plays an important role in protein translocation process. Second, a highly hydrophobic H-domain arising from a stretch of leucines was needed for efficient processing of the signal peptide and translocation of the protein. Third, a favored signal peptide cleavage motif “A-X-A” at the end of the C-domain was observed. These observations in high secretory efficiency signal peptides may indicate a new avenue for the rational optimization of signal peptides for the secretion of heterologous proteins. Systematic optimization of optimal signal peptides can be a powerful strategy to promote secretory production of keratinase in different heterologous expression systems.

5.3 Pro-peptide engineering

Since the pro-peptide region was found to play a major role in assisting the maturation process and significant structural rearrangement upon pro-peptide autoprocessing, a new approach, termed "pro-peptide engineering", was developed as a useful method to enhance the production and catalytic properties of autoprocessing proteases (Takagi & Takahashi, 2002; Gu et al., 2019). It may directly catalyze the folding reactions or facilitate processes such as structural organization and oligomerization, localization, and sorting. Accordingly, a series of modifications have been conducted, focusing on the pro-peptide of keratinase to improve yield and catalytic properties. Sharma et al. found the N-terminal region in keratinase KerP was considerably variable with an alignment percentage of just 54%. Though variable, the N-terminal region however contained interspersed stretches of conservation, thus truncations were made randomly to study the effect of deletions in the N-terminal pro-sequence. With the extension of truncated length, the enzyme activity decreased gradually. By gradual truncating, they identified the chaperone activity of N-terminal pro-peptide and revealed that only a 187-bp pro-sequence region was the minimum length required for the correct folding of keratinase KerP into its active conformation. The size of pro-sequence had a direct impact on the conformation of protein, hence leading to altered kinetics of enzyme (Sharma et al., 2011). The pro-sequences of keratinases from *Bacillus licheniformis* (KerBL) and *Bacillus pumilus* (KerBP) were

swapped, and the mutants showed altered pH and temperature kinetics as well as substrate specificities, indicating that the pro-sequence could mediate conformational changes (Rajput et al., 2012). Site-directed mutagenesis around the N-terminal pro-sequence of keratinase Sfp2 improved the specific activity and protein yield nine-fold (Li et al., 2013a). Since pro-peptide must be cleaved to release active mature keratinase, cleavage efficiency tends to be a crucial rate-limiting step. To enhance production of keratinase, Liu et al. performed single amino-acid substitutions, single residue deletions, and linker introductions at the cleavage site of the pro-peptide, resulting in a 50% increase in mature enzyme yield (Liu et al., 2014b). Moreover, Su et al. achieved significant improvement in keratinase activity, from 179 to 1114 U/mL, and altered catalytic properties by combining amino acid substitutions at the cleavage site and performing multisite saturation mutagenesis of the pro-sequence (Su et al., 2019). These results indicate that pro-sequence engineering, in the form of site-directed mutagenesis, swapping, chimeras, and gene shuffling, is a promising technology for improving productivity and creating unique mutants with various beneficial properties.

5.4 C-terminal engineering

It has been reported that some unnecessary amino acids or domains exist at the C-terminal of enzymes, affecting catalytic efficiency and enzymatic properties. Therefore, C-terminal modifications have been explored as a novel protein engineering strategy (Yang et al., 2013). Studies have found that partially truncating the C-terminal sequence in keratinase can improve catalytic efficiency, thermophilicity, salt tolerance, and detergent tolerance (Fang et al., 2016a). This sheds light on the functions of the C-terminal domain, indicating an available strategy for keratinase protein engineering to improve catalytic efficiency and other properties.

5.5 Catalytic center modification

The catalytic center and the surrounding substrate binding pocket are the most essential domains for a protease (Giger et al., 2013). Combining *in silico* analysis and site-directed mutagenesis around the catalytic center has been extensively adopted to

improve catalytic efficiency. This powerful strategy can obtain highly active variants with less effort using calculations and predictions (Romero-Rivera et al., 2016). The crucial role of Leu31 and Thr33 in keratinase from *B. pumilus* CBS was probed in terms of substrate recognition and catalytic ability by site-directed mutagenesis (Jaouadi et al., 2014). Through computational analysis of the simulated 3D structure, it was found that Thr33 was directly involved in the substrate binding site S2, and Leu31 was involved in a network of hydrophobic interactions that may shape the active site of keratinase. As a result, the binding pocket and catalytic cavity of the mutants have been enlarged, which is anticipated to better accommodate large side chains, and to be more selective, thus altering the substrate specificity. Similarly, from *in silico* analysis of the homologous model, four residues lying at the entrance or bottom of the S1 pocket of keratinase KerSMD were deduced to be related to substrate specificity (Fang et al., 2015). By mutating these residues, the S1 pocket was enlarged, and the catalytic ability was improved and shifted to hydrolyze macromolecular substrates. These findings provide insight into the relationship between the catalytic center binding pocket, catalytic ability, and substrate specificity in keratinase.

5.6 Combination of multiple strategies

The combination of multiple mutation strategies may have a superposition effect through cooperative action, especially with regard to keratinase, the functions of whose pro-peptide, C-terminal domain, and catalytic center have been investigated. Fang et al. improved the catalytic ability and thermostability of keratinase KerSMD by replacing the pro-peptide and C-terminal domain with those of a homologous protease, KerSMF, which exhibits higher catalytic efficiency (Fang et al., 2016b). N-pro-peptide replacement increased activity towards casein by more than 2-fold, the C-terminal domain replacement notably improved keratinase activity, and the replacement of both N- and C-terminal domains generated a mutant with enhanced catalytic efficiency and enzymatic properties. Moreover, the group further mutated the binding pocket of the variant based on analysis of the homology model structure,

combined with the C-terminal fusion strategy (Fang et al., 2017). Site-directed mutagenesis of binding sites improved extracellular keratinolytic activity and thermostability, and the C-terminus fusion created a new variant with the highest substrate specificity and keratinolytic activity. The researchers also combined the N-terminal pro-peptide replacement with site-directed mutagenesis in a catalytic center to enhance keratinase activity and production (Fang et al., 2019). This comprehensive mutation improved specific activity and extracellular enzyme yield. These results indicate that simultaneously creating multiple mutations can be adopted as an effective method to improve and customize keratinases.

6. Summaries and prospects

The first report of keratinase occurred decades ago, and a number of keratinases have been identified in subsequent studies. Their specific catalytic abilities towards rigid keratin substrates and in diverse applications have been widely recognized. However, industrial production and commercialization of keratinase lag the available information due to their low productivity and poor catalytic properties. A boost from molecular biotechnology, including gene and protein engineering, has generated a number of enzymes. These powerful techniques and strategies are the impetus for development of keratinase for industrialization and commercialization. In this critical review, we present an overview of the outstanding catalytic performance of keratinases, and further provide comprehensive insight into the gene sequences and protein structures of keratinase. Developments and current advances in keratinase heterologous expression in *E. coli*, *Bacillus*, and yeast systems are summarized. We also discuss protein engineering strategies focused on signal peptide, pro-peptide, C-terminal domain, and catalytic center of keratinases, demonstrating that protein domains such as signal peptides and pro-peptides play critical roles in keratinases. Engineering of these domains is an important strategy to increase expression of keratinases, which can generate synergistic effects with catalytic center modifications.

In addition to protein domains discussed in this review, other factors are also important for enzyme activity and stability, such as flexible sites (Yu & Huang, 2014),

enzyme access tunnels (Kokkonen et al., 2019), and enzyme microenvironments (Lancaster et al., 2018). These non-central portions have tremendous potential to further improve efficiency and capabilities of keratinase. In addition, computer-aided rational or semi-rational modifications of keratinases will become an important area of research using various advanced *in silico* forecasting and analysis tools (Ebert & Pelletier, 2017; Frushicheva et al., 2014). Taking full advantage of these powerful tools, we may even design and create an all-around keratinase, capable of digesting desired substrates with desired efficiency (Lu et al., 2009; Rittle et al., 2019).

It is expected that the information associated with keratinase heterologous expression and protein engineering provided by the present review are valuable for the development and further study of keratinase, and will help accelerate their industrial production and commercialization.

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Fig. 1 Summary of reviews on keratinases in chronological order.

Fig. 2 Catalytic performances and application potentials of keratinase in feed, fertilizers, plant culturing, leathering, cosmetics, and medicines.

Fig. 3 The degradation of keratin substrates by keratinase producing microorganisms. Degradation process including the reduction of disulfide bonds with sulfides, reductase, or keratinase as reductant; and the hydrolysis of polypeptide chains by keratinase.

Fig. 4 Gene mining strategies. A: Conventional mining via screening and isolation of keratinase-producing microorganisms. B: Metagenomics mining, based on the construction of metagenomic libraries. C: Mining of existing genetic database.

Fig. 5 Crystal structure of reported keratinases. A. rMtaKer reported by Wu et al. (Wu et al., 2017). The structure is recorded in PDB (No. 5WSL). Mature rMtaKer is made of a seven-stranded parallel β sheet flanked by six α -helices (orange) and five β -sheets (cyan), containing two calcium ion binding sites. The catalytic triad residues (Asp, His and Ser) are highly conserved with AquI (PDB ID: 4dzt), VPR (PDB ID: 1sh7), Carlsberg (PDB ID: 1cse), KerA (PDB ID: 4gi3) and Tk-subtilisin (PDB ID: 2z2x); Tyr282 seals the hydrophobic pocket of S1 site along with several hydrogen bonds around the active site. B. A keratinolytic enzyme from *Fervidobacterium pennivorans* (Kim et al., 2004). The protease is composed of four domains: a catalytic domain (CD), two β -sandwich domains (SDs), and the propeptide (PD) domain. C: The mature part embraces the PD, which fits into the curved surface of CD and blocks the substrate binding sites. The activation of mature part needs the cut of PD.

Fig. 6 The maturation process of keratinase including the synthesis, secretion, folding, and activation.

Fig. 7 Homology structure model of keratinase KerSMD. A: Alignment between KerSMD (yellow) and its mutant (purple) structures (Fang et al., 2016b). B: Alignment of the residues of the S1 pockets of KerSMD (yellow) and its mutant (purple). C: The homology structure of KerSMD showing the catalytic triad residues (green) and S1 pocket residues (yellow) (Fang et al., 2015). D: The S1 pocket shown on the model surface, with the nitrogen, oxygen, and carbon atoms labeled in blue, red, and yellow.

Table 1 Reported keratinase genes and proteins in NCBI, complete CDS.

GenBank No.	Source	Length	Protein Accession	Amino Acid Residues	Catalytic Residues	Characteristic	Reference
DQ071570.1	<i>B. licheniformis</i> strain MKU 3	1140	AA082467.1	379	137D, 168H, 325S	A maximum keratinolytic activity of 166.2 U/mL in <i>B. megaterium</i> using keratin azure as substrate	(Radha & Gunasekaran, 2007)
JF800188.1	<i>Bacillus</i> sp. MKR1	1140	AEI59720.1	379	137D, 168H, 325S	Intracellular keratinase with activity of 200 U/mL using keratin azure as substrate	(Dabbagh et al., 2011)
MG738728.1	<i>Bacillus</i> sp. LCB12	1140	AY003504.1	379	-	Keratinase activity of 1,332 U/mL in <i>B. subtilis</i> using wool keratin as substrate. Activity completely inhibited by PMSF, while slightly inhibited by EDTA	(Tian et al., 2019a)
MF327392.1	<i>B. licheniformis</i> strain Y6	1140	AWF79184.1	379	137D, 168H, 325S	Thermophilic keratinase with a keratinase activity of 437 U/mL	(Liu et al., 2018)

						in <i>E.coli</i> using casein as substrate	
JX50468 1.1	<i>B. licheniformis</i> strain BBE11-1	1140	AFT9204 0.1	379	137D, 168H, 325S	Extreme alkaline, oxidation-resi stant keratinase with 323 U/mL activity in <i>B.subtilis</i> using soluble keratin as substrate	(Liu et al., 2013a)
KJ66154 2.1 (<i>kerA</i>)	<i>B. licheniformis</i> strain MZK05	1140	AIH1356 6.1	379	137D, 168H, 325S	Crude keratinase alone (without chemicals) can dehair the goat skin completely, showing potential in dehairing of leather	(Nahar et al., 2016)
EU50284 4.1 (<i>kerRP</i>)	<i>B. licheniformis</i> strain RPK	1457	ACA9799 1.1	379	137D, 168H, 325S	Maximum protease activity (4150 U/mL with casein as a substrate and 37.35 U/mL with keratin as a substrate)	(Fakhfakh et al., 2009)
AY59014 0.1	<i>B. licheniformis</i> strain RG1	1140	AAS8676 1.1	379	137D, 168H, 325S	Extracellular protease with an activity of 425 U on feather substrate	(Ramnani et al., 2005)
FJ619651	<i>B. pumilus</i>	1199	ACM4773	383	140D,	44-130 bp:	(Fakhfakh-Z

.1	strain A1	5.1			172H, 329S	sig-peptide 131-367 bp: pro-peptide 368-1192: mature keratinase Serine Metallokeratinase	ouari et al., 2010)
HM2191 83.1	<i>B. pumilus</i> strain KS12	1152	ADK1199 6.1	383	140D, 172H, 329S	Thermostable alkaline serine protease with a high specific keratinase activity of 460 U/mg in <i>E.coli</i> using powdered chicken feather as substrate	(Rajput et al., 2011)
KP69422 1.1	<i>B. tequilensis</i> strain Q7	1343	ATCC 2021 9.1	383	140D, 172H, 329S	Serine neutral keratinase with 1260 U/mg activity in <i>E.coli</i> using keratin azure as a substrate	(Jaouadi et al., 2015)
KF15296 6.1 (<i>kerUS</i>)	<i>B. brevis</i> strain US575	1343	AGO5846 6.1	383	140D, 172H, 329S	Serine alkaline keratinase with 20,000 U/mg activity in <i>E.coli</i> using keratin azure as substrate	(Jaouadi et al., 2013)
KX18483 1.1	<i>B. pumilus</i> strain C4	1152	ANQ6833 3.1	383	140D, 172H,	Serine keratinase	(Fellahi et al., 2016)

(<i>ker1</i>)					329S	with the ability to degrade both alpha and beta keratin	
KX18483	<i>B. pumilus</i>	1152	ANQ6833	383	140D,	Serine	(Fellahi et
2.1	strain C4		4.1		172H,	protease with	al., 2016)
(<i>ker2</i>)					329S	keratinase activity	
KC62129	<i>B. circulans</i>	1168	AGN9170	383	140D,	Thermo and	(Benkiar et
4.1	strain DZ100		0.1		172H,	detergent	al., 2013)
(<i>sapDZ</i>)					329S	stable alkaline serine keratinolytic protease with a specific activity of 75,000 U/mg after purification using keratin azure as substrate	
AM7487	<i>Bacillus</i>	1268	CNC0304	383	140D,	Detergent-sta	(Jaouadi et
27.1	<i>pumilus</i> CBS		0.1		172H,	ble serine	al., 2008)
(<i>sapB</i>)					329S	alkaline protease with keratin hydrolysis activity	
KM4944	<i>B. subtilis</i>	1342	AIY6281	362	119D,	Keratinase	(Gupta et al.,
93.1	strain		2.1		151H,	activity of	2017)
(<i>kerC</i>)	RSE163				308S	450 ± 10.43 U in <i>E. coli</i> using chicken feather as substrate	
KR86899	<i>B.</i>	1368	AKR0513	382	139D,	Extreme	(Yang et al.,
6.1	<i>amyloliquefac</i>		4.1		171H,	alkaline	2016)
(<i>kerK</i>)	<i>iens</i> strain K11				328S	keratinase that degrade insoluble keratin	

						substrates with 1500 U/mL keratinolytic activity using feather keratin powder as substrate	
KJ78344 4.1	<i>G. stearothermo philus</i> strain AD-11	1641	AJD7742 9.1	546	-	Zn-dependent metalloprotease; belong to Peptidase M4 family includes thermolysin, showing specific keratinolytic activity of 1437.6 U/mg after being purified	(Gegeckas et al., 2015)
LC02945 3.1 (<i>kerN4</i>)	<i>S. maltophilia</i>	1770	BAC2663 2.1	589	86D, 125H, 365S	Thermostable serine keratinase with three calcium binding sites: calcium binding site 1: (37,95,139) calcium binding site 2: (258,288) calcium binding site 3: (266,268,271,273)	(Jankiewicz et al., 2016)
HM5906 50.1	<i>S. maltophilia</i> strain	1818	ADK7482 8.1	580	177D, 237H,	Alkaline serine	(Zou et al., 2011)

(<i>kerF</i>)	YHYJ-1				409S	protease with specific keratinase activity of 726 U/mg using soluble keratin as substrate, showing excellent feather degradation activity		
FJ765514.1 (<i>kerD</i>)	<i>S. maltophilia</i> strain YHYJ-1	1905	ACN82379.1	634	209D, 272H, 456C	Neutral protease with keratinase activity of 10.5 U/mL in <i>E.coli</i>	(Ji et al., 2011)	

Table 2 Heterologous expression status of keratinase in *E.coli* expression systems.

Gene	Source	Plasmid	Host	Substrate	Activity (U/mg)	M. W. (kDa)	Temp. (°C)	pH	Reference
<i>ker</i>	<i>P. aeruginosa</i>	pET-43b(+)	<i>E. coli</i> AD494(DE3)pLysS	Azo-keratin	146	33	10-60	6.0-9.0	(Lin et al., 2009a)
<i>kerBL</i>	<i>B. licheniformis</i> ER-15	pEZZ18	<i>E. coli</i> HB101	Keratin azure	75	28	60	8.0-12.0	(Tiwari & Gupta, 2010a)

<i>kerP</i>	<i>P. aeruginosa</i>	pEZZ18	<i>E. coli</i> HB101	Chicken feather	3700	33	$t_{1/2}$ =12 min (70°C)	4.0-12.0	(Sharma & Gupta, 2010)
<i>rK27</i>	<i>B. pumilus</i> KS12	pEZZ18	<i>E. coli</i> HB101	Chicken feather	1240	27	30-90 (70°C)	7.0-10.0 (9.0)	(Rajput et al., 2011)
<i>kerA</i>	<i>B. licheniformis</i> S90	pET30a	<i>E. coli</i>	Keratin azure	26.8 U/m	45	50	7.5	(Hu et al., 2013)
		pET32a			19.4 U/m			8.5	
<i>kerU</i>	<i>B. brevis</i>	pTrc99A	<i>E. coli</i> BL21	Chicken feather	2000	29	40	8.0	(Jaouadi et al., 2013)
<i>S</i>	<i>US575</i>	A			0				
<i>kerS</i>	<i>S. maltophilia</i>	pET22a	<i>E. coli</i> BL21 (DE3)	Soluble keratin (1%, w/v)	350 U/m	48	$t_{1/2}$ =64 min (60°C)	8.0	(Fang et al., 2014)
<i>MD</i>						40		9.0	
<i>kerS</i>	BBE11-1				L				
<i>MF</i>					712 U/m				
					L				
<i>kerE</i>	<i>B. licheniformis</i> BBE11-1	pET-22b (+)	<i>E. coli</i> BL21 (DE3)	Keratin (1%)	1206	30	50	10.0	(Liu et al., 2014a)
<i>kerA</i>	<i>B.</i>	pGEX-	<i>E. coli</i> BL21	Keratin-	1647	39	-	-	(Naha

	<i>licheniformis</i> MZK-05	6p-2		azure					ret al., 2016)
<i>ker</i>	Soil samples metagenome	pET-28 a(+)	<i>E. coli</i> BL21 (DE3)	Soluble keratin (1%, w/v)	120 U/m L	26	40–60 (55)	5.0–12.0 (10.0)	(Su et al., 2017)
<i>ker</i>	<i>B. subtilis</i> RSE163	pSPOR T	<i>E. coli</i> Mach 1	Feather powder	450	46	-	-	(Gupta et al., 2017)
<i>KBAL T</i>	<i>B. altitudinis</i> RBDV1	pCold-TF	<i>E. coli</i> BL21	Keratin azure	1986	43	85	8.0	(Pawar et al., 2018)
<i>kerT1</i>	<i>Thermoactinomyces</i> sp. YT06	pET29 a	<i>E. coli</i> BL21 (LE ⁺)	Keratin	1325	28	50-60 (65)	6.0-1.0 (8.5)	(Wang et al., 2019)
<i>ker02</i>	<i>Bacillus</i> sp. JM7 (From deep sea)	pCold	<i>E. coli</i> BL21(DE3)	Feather powder (0.2%, w/v)	123.4	38	30-60 (40-50)	5.0-1.0 (7.0-9.0)	(Jin et al., 2019)

Table 3 Heterologous expression status of keratinase in *Bacillus* expression systems.

Gen	Source	Plasmid	Host	Substrate	Activity	M. W.	Temp.	pH	Reference
e					(U/mg)	(kD)	(°C)		

					g)		a)		
<i>kerA</i>	<i>B.licheniformis</i>	pUB18-p43	<i>B. subtilis</i>	Azo-ke	120	-	-	-	(Lin et al., 1997)
			DB104	U/m	L				
<i>rK</i>	<i>P. aeruginosa</i>	pRPA	<i>B. subtilis</i>	Azo-ke	994.8	33	10-50	6.0-9.0	(Lin et al., 2009b)
			DB104	U/m			(60)	(8.0)	
<i>ker</i>	<i>B. licheniformis</i>	pMA0911	<i>B. subtilis</i>	Soluble keratin	323	30	10-50	7.0-11.5	(Liu et al., 2013a)
			WB600	(1%, w/v)	L		(40)	(10.5)	
<i>rK2</i>	<i>B. pumilus</i>	pSTREPHI	<i>B. subtilis</i>	Feather powder	38.1	27	-	-	(Rajput et al., 2014)
7	KS12	S1525	WB980						
<i>kerB</i>	<i>B. licheniformis</i>	pSTOP162	<i>B. subtilis</i>	Keratin	1286	30	40	10.0	(Liu et al., 2014a)
		2	WB600	(1%)					
<i>kerK</i>	<i>B. amyloliquefaciens</i>	pUB110	<i>B. subtilis</i>	Feather keratin	-	27	50	6.0-12.0	(Yang et al., 2016a)
			SCK6	powder				(11.0)	
<i>kerP</i>	<i>B. polyfermenticus</i>	pL MT	<i>B. subtilis</i>	Feather powder	473 ± 20	26	60	9.0	(Dong et al., 2017)
			PT5	U/m	L				
<i>kerB</i>	Soil metagenomes	pMA5	<i>B. subtilis</i>	Soluble keratin	164.8	-	60	6.0-12.0	(Tao et al., 2018)
			WB600	(1%, w/v)	L				
<i>kerT</i>	<i>B.</i>	pLY	<i>B.</i>	Soluble	1535	28	60	10.0	(Cao et al., 2018)

	<i>amyloliquef</i>		<i>subtilis</i>	keratin	U/m				al.,
	<i>aciens</i>		WB600	(1%,	L				2019)
	TCCC1131			w/v)					
9									
<i>ker</i>	<i>Bacillus sp.</i>	pMA0911	<i>B.</i>	Wool	9813.	30.	60	10.0	(Tian et
	LCB12		<i>subtilis</i>	keratin	2	95			al.,
			SCK6	(2%,					2019)
				w/v)					
<i>KER</i>	<i>B. subtilis</i>	pSUGV4	<i>B.</i>	Keratin	1300	36	50	9.0	(Yong et
71	S1-4		<i>subtilis</i>	powder		1			al.,
			WB600						2020)
<i>ker</i>	<i>B.</i>	pWH1520	<i>B.</i>	Keratin	33.25	30	-	-	(Radha
	<i>licheniformi</i>		<i>megate</i>	azure	(166.				&
	<i>s</i> MKU3		<i>rium</i>		2				Gunasek
			ATCC		U/m				aran,
			14245		L)				2007)
<i>ker</i>	<i>B.</i>	pWH1520	<i>B.</i>	Keratin	17.25	30	-	-	(Radha
	<i>licheniformi</i>		<i>megate</i>	azure					&
	<i>s</i> MKU3		<i>rium</i>						Gunasek
			MS941						aran,
									2008)

Table 4 Heterologous expression status of keratinase in Yeast expression systems and non-universal expression host.

Gene	Source	Plasmid	Host	Substrate	Activity	M. W.	Temp.	pH	Reference
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					(U/mg)	(kDa)	(°C)		
ker	<i>A. fumigatus</i>	pHILD-2	<i>P. pastoris</i> SMD 1165	Keratin-azure	1.3 U/mL	31	-	-	(Noronha et al., 2002)
rK	<i>P. aeruginosa</i>	pPICZ RC	<i>P. pastoris</i> SMD1168H	Azo-keratin	1003 U/mL	33	10-6	6.0-9	(Lin et al., 2009c)
ker	<i>B. licheniformis</i> S90	pPICZ αA	<i>P. pastoris</i>	Keratin-azure	324 U/ml	~39	50	7.5	(Hu et al., 2013b)
ker	<i>B. licheniformis</i> BBE11-1	pPIC9 K	<i>P. pastoris</i> GS115	Keratin (1%)	1245	38	70	10.0	(Liu et al., 2014a)
ker	<i>B. amyloliquefa</i> ciens K11	pUB11 0	<i>B. amyloliquefa</i> ciens K11 (Parent strain)	Feather keratin powder	1500 U/mL	-	-	-	(Yang et al., 2016)
ker	<i>B. licheniformis</i> PWD-1	pUC57	Insect <i>Spodoptera frugiperda</i> (Sf9) cells	Azo-keratin	635	38	45	8.0	(Huang et al., 2017)

Note: The activity with the unit of “ U/mg ” represents the specific activity of purified keratinase, the unit of “ U/mL ” represents the crude enzyme.

The temperature and pH in bracket represent the optimal condition when the stability range is given.

Table 5 Protein engineering strategies to improve the keratinase production, activity, and thermostability.

	Keratinase	Source	Express ion System	Modification	Effect/Conclusion	Reference
Signal peptide	-	<i>Bacillus sp.</i>	pMA091	Signal peptide	1.2-fold higher activity: signal peptide optimization can enhance the extracellular keratinase activity.	(Tian et al., 2019)
		LCB12	<i>subtilis</i>	optimization		
			SCK6	on		
Pro-peptide	KerP	<i>P. aeruginosa</i>	pEZZ-1	Truncation of N-terminal pro-peptide	Identification of region with chaperone activity: the size of the pro-sequence has a direct impact on the conformation of the protein hence leading to altered kinetics of the enzyme.	(Sharma et al., 2011)
	KerBL	<i>B. licheniformis</i>	p ⁺ ZZ13	Swapping of pro-sequence	Ker ProBP-BL showed more thermostable with a $t_{1/2}$ of 45 min at 80°C contrary to not stable beyond 60°C; Increased K:C ratio by twelve-fold; Ker ProBL-BP revealed altered pH and temperature kinetics: Pro-sequence mediated conformational changes.	(Rajput et al., 2012)
	KerBP	<i>B. pumilus</i>	HB101			
	KerA	<i>B. licheniformis</i>	pMA5/ <i>B. subtilis</i>	pro-peptide engineering	Mature keratinase yield of Leu(P1)Ala mutant increases 50%: optimizing the cleavage	(Liu et al., 2014b)

	BBE11-1	WB600	g:	site of pro-peptide will affect	
			Single	the cleavage efficiency of	
			residue	pro-peptide, and the mature	
			substitutio	enzyme yield.	
			ns;		
			Single		
			residue		
			deletions;		
			Linkers		
			introductio		
			n		
KerSfp2	<i>S. fradiae</i>	pJTU48	Amino	Specific activity of L(-1)F	(Li et
	var. k11	82/ <i>S.</i>	acid	mutant was improved by nine	al.,
		<i>lividans</i>	substitutio	times; the yield of L(-1)F	2013a)
		1326	positions	mutant was	
			-1 and -2	nine time of total protein and	
			of	twice of culture supernatant:	
			pro-peptid	replacement of the residue at	
			e	the cleavage site of	
				keratinase may be an	
				effective approach for	
				obtaining a higher yield.	
KerBp	Soil	pMA5/	Amino	Substitutions at the pro-peptide	(Su et
	metageno	<i>B.</i>	acid	cleavage site (P1) result in a	al.,
	mes	<i>subtilis</i>	substitutio	3-fold activity increase;	2019)
		WB600	ns at	multisite saturation	
			cleavage	mutagenesis achieves a	
			site of	significant improvement of	
			pro-peptid	keratinase activity from 179 to	

Mature Keratinase				e;	1114 U/mL and alternants	
				Site-direct	catalytic properties:	
				ed	Pro-peptide engineering	
				mutagenes	played a crucial role in high	
				is of the	expression and engineering	
				pro-peptid	of keratinase.	
				e		
	SAPB	<i>B. pumilus</i>	pBJ/ <i>B. subtilis</i>	Site-direct	Higher keratinolytic activity:	(Jaouad
				ed	Leu31 and Thr 33 are of	i et al.,
	CBS		DB430	mutagenes	Crucial role for shaping the	2014)
				is in the	binding pocket, thus altering	
				binding	the substrate specificity.	
				pocket		
	KerSM	<i>S. maltophili</i>	pET22b/ <i>E. coli</i>	Site-direct	Shift the catalytic ability to	(Fang
	D	<i>a</i>	BL21	ed	hydrolyze synthetic peptides	et al.,
			(DE3,	mutagenes	and macromolecular	2015)
				is in the	substrates;	
				S1 pocket	improved the keratinolytic	
					activities;	
					enhanced thermostability:	
					steric hindrance and	
					hydrophilia in the S1 pocket	
					have a significant effect on	
					catalytic preference, and the	
					hydrogen bonds in the S1	
					pocket have great influence	
					on the thermostability.	
	WF146	<i>Bacillus sp.</i>	pHS/ <i>E.coli</i>	Introducti	Increased the autolysis	(Liang
				on of	resistance of the enzyme under	et al.,

C-terminal		WF146	BL21(D E3)	prolines into the autolytic sites (N63P and A66P)	reducing conditions; a 2.8-fold longer half-life at 80°C; higher hydrolytic activities at high temperatures: N63P/A66P is of great interest to be used in stabilizing the enzyme against autolysis.	2010)
	KerSM D	<i>S. maltophila</i> <i>a</i>	pET22b/ <i>E. coli</i> BL21 (DE3)	Partially truncation of PPC domain (Pre-peptidase C-terminal domain)	Improved catalytic efficiency to the synthetic substrate AAPF; obviously decreased collagenase activity; enhanced thermophilicity; improved tolerance to alkalinity, salt, chaotropic agents, and detergents: shine light on the function of the C-terminal domain and provided a useful strategy to improve specific activity and catalytic efficiency.	(Fang et al., 2016a)
	KerSM D	<i>S. maltophila</i> <i>a</i>	pET22b / <i>E. coli</i> BL21 (DE3)	Replacement of the N-terminal domain; Replacement of the C-terminal domain;	two-fold increased catalytic activity towards casein	(Fang et al., 2016b)
Combination of multiple	KerSMF	<i>a</i> BBE11-1	BL21 (DE3)	N-terminal domain; Replacement of the C-terminal domain;	54.5% increased k_{cat}/K_m value on a synthetic peptide, succinyl-Ala-Ala-Pro-Phe-p-nitroanilide; enhanced thermostability; high activity under mesophilic	

			Replacem	conditions: C-terminal	
			ent of both	domain and N-pro-peptide	
			the N- and	are important for substrate	
			C-terminal	specificity, activity and	
			domain;	thermostability.	
			Deletion		
			of the		
			C-terminal		
			domain		
KerSM	<i>S.</i>	pET22b	Site-direct	Higher extracellular	(Fang
D	<i>maltophili</i>	<i>/ E. coli</i>	ed	keratinolytic activity and	et al.,
	<i>a</i>	BL21	mutagenes	specific activity; enhanced	2017)
	BBE11-1	(DE3)	is of	thermostability:	
			binding	It is highly effective to obtain	
			pocket;	excellent keratinases by	
			C-terminu	site-directed mutagenesis	
			s fusion	and C-terminus fusion based	
				on model structure analysis.	
KerSM	<i>S.</i>	pET22b	N-terminal	Achieved 2.5-fold	(Fang
D	<i>maltophili</i>	<i>/ E. coli</i>	pro-peptid	improvement of extracellular	et al.,
	<i>a</i>	BL21	e	enzyme yield; improved more	2019)
	BBE11-1	(DE3)	replaceme	than 1300 U/mg of specific	
			nt;	activities: exchange of	
			Site-direct	N-terminal	
			ed	Pro-peptide and site-directed	
			mutagenes	mutagenesis in S1 pocket can	
			is in	dramatically improve the	
			catalytic	extracellular specific activity	
			domain	and keratinolytic protease	

				production.	
KerA	<i>B.</i>	pMA5/	Folding	An 8.6-fold increase in the $t_{1/2}$	(Liu et
	<i>lichenifor</i>	<i>B.</i>	free	value at 60°C;	al.,
	<i>mis</i>	<i>subtilis</i>	energy	an approximately 5.6-fold	2013b)
	BBE11-1	WB600	change	increase in catalytic efficiency:	
			(DDG)	PoPMuSiC algorithm in	
			prediction	combination with molecular	
			by	modification can be applied	
			PoPMuSi	to enhance the thermostability	
			C	and catalytic efficiency.	
			algorithm;		
			Amino		
			acid		
			substitutio		
			ns

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 6

Fig. 7

Figure 1

Figure 2

Figure 3

Figure 4

A

Conserved catalytic triad residues

Hydrophobic pocket of S1 site around active site

Mature rMtaKer monomers

Meiothermus taiwanensis WR-220 MtaKer
Thermus aquaticus AquI
Vibrio sp. PA-4 VPR
Bacillus subtilis Carlsberg
Bacillus licheniformis KerA
Thermococcus kodakarensis KOD1 TK-subtilisin

B

C

Figure 5

Figure 6

Figure 7