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Review

A Review: Biodegradation and Applications of Keratin Degrading Microorganisms and Keratinolytic Enzymes, Focusing on Thermophiles and Thermostable Serine Proteases

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Abstract: Keratins are hard-degrading fibrous proteins, insoluble in water and organic solvents, often accumulated in nature and major components in feathers, skins, hair, horn, nail, hoof etc., Keratin-degrading microorganisms such as bacteria, archaea, actinomycetes and fungi employ keratinases to attack keratin. Keratinases belonging to subtilisin-like serine proteases were classified based on similarity of amino acid sequences. Keratinolytic thermophilic or hyperthermophilic bacteria and archaea have been known to degrade keratin at ≥70°C. General properties of thermozymes such as stability to heat and resistance to denaturing conditions; e.g., solvents and detergents have drawn attention to various biotechnological industries. Some bacterial and archaeal keratinases degrade not only fibrous keratin but also digest recalcitrant prion proteins, an etiologic agent of spongiform encephalopathies of brain and nervous system. Keratin and keratinase have a number of applications in various sectors, i.e., biotechnology, cosmetic and pharmaceutical industries, medical therapy and waste management. On the other hand, accumulation of excess amount of keratin is recognized as solid waste and troublesome environmental pollutant. Biodegradation involving either keratinolytic thermophiles or thermostable keratinases not only improve digestibility and nutritive values of keratinous meals (for mixers in animal feed stuffs), but also minimize risk from infection and microbial toxin transmitted to livestock.

Keywords: Keratinase, Keratin, Thermophiles, Waste Management, Biodegradation

Introduction

Keratins are hard-degrading fibrous proteins that can be found as major components in feathers, skins, hair, horn, nail, etc. Keratinase is a particular class of extracellular subtilisin-like serine protease with a capability of degrading insoluble keratin substrates. The enzymes hydrolyze the disulfide (-S-S-) and peptide bonds of keratinous substrates and are widely produced mostly by bacteria, archaea and fungi. Enzymes from thermophiles and hyperthermophiles are often referred to as thermozymes. Thermozymes have striking characteristics such as active at high temperature, stable to heat and resistant to denaturing conditions; e.g., solvents and detergents. Many thermostable keratinases were found more active in the presence of Sodium Dodecyl Sulfate (SDS). Thermozymes are attractive to industries and preferably used when the enzymatic process is compatible with high-temperature process conditions. The main advantages of performing processes at high temperatures are to reduce risk of microbial contamination, lower viscosity, reduce mixing speed, improve transfer rates and increase substrate solubility (Vetriani *et al*., 1998; Niehaus *et al*., 1999).

Thermostable keratinases and some related subtilisin-like serine proteases from thermophilic bacteria (e.g., *Thermoanaerobacter* and *Fervidobacterium*) displayed their maximal activities at high temperature range (60-100°C); and hyperthermophilic archaea (e.g., *Aeropyrum*, *Thermococcus* and *Pyrococcus*) displayed their maximal activities at even higher (90-110°C) (Fiala and Stetter, 1986; Friedrich and Antranikian, 1996). Keratin and keratinase as well as keratinolytic microorganisms have tremendous applications in various sectors including

biodegradation and waste management, biotechnology and industry, cosmetic and pharmaceutical products and medical treatments (Mitsuiki *et al*., 2006; Šnajder *et al*., 2012; Suzuki *et al*., 2006; Gupta *et al*., 2013). This review presents keratinase producing microorganisms with an emphasis on thermophiles. Biochemical properties and phylogenetic of thermostable serine proteases, particular to the fervidolysin and islandisin from *Fervidobacterium* spp., as well as activity of some bacterial keratinases on prions were highlighted. Applications of keratin and keratinase and feasibility of biodegradation involving keratin hydrolysis by thermophiles were discussed.

Sources of Keratinases

Mesophilic Bacteria

Keratinases are widely produced by organisms belonging to the domains Eukarya, Bacteria and Archaea. The enzymes mainly attack the disulfide and peptide bonds of keratinous substrate. Keratinophilic bacteria, particularly from the genus *Bacillus* and filamentous bacteria belonging to actinomycetes were the most frequent isolates; for examples, *Bacillus licheniformis* PWD-1, *B. licheniformis* KK1, *B. subtilis* S14, *B. subtilis* FDB-29, *Bacillus* sp. MSK103, *Streptomyces* sp. S.K.1-02, *Streptomyces* sp. BA7, *Actinomadura keratinilytica* Cpt29 and *Nocardiopsis* sp. TOA-1 (Williams *et al*., 1990; Lin *et al*., 1992; Letourneau *et al*., 1998; Wang and Shih, 1999; Korkmaz *et al*., 2003; Gousterova *et al*., 2005; Mitsuiki *et al*., 2006; Yoshioka *et al*., 2007; Habbeche *et al*., 2014).

Thermophilic Bacteria

To date, few thermophilic and extremely thermophilic bacteria known to produce keratinases include *Thermoanaerobacter keratinoplilus*, *Thermoanaerobacter* sp. strain 1004-09, *Fervidobacterium pennivorans*, *F. islandicum* AW-1, *F. thailandense* FC2004^T and *Fervidobacterium* sp. FA004. Keratinases from *T. keratinoplilus* and the strain 1004-09 active optimally at 85°C, pH 8 and 60°C, pH 9.3, respectively. The enzyme from the strain 1004-09 had a broad temperature range from 20-92°C and was stimulated by SDS. Keratinases from *F. pennivorans*, *F. islandicum* AW-1 and the strain FA004 exhibited optimal activities between 80-100°C. The enzymes hydrolyzed casein and keratin in the presence of SDS and were extremely stable at 70 and 80°C for days (Friedrich and Antranikian, 1996; Riessen and Antranikian, 2001; Nam *et al*., 2002; Kublanov *et al*., 2009; Keawram *et al*., 2016; Kanoksilapatham *et al*., 2016).

Hyperthermophilic Archaea

Hyperthermophilic archaea such as *Thermococcus* and *Pyrococcus* of the order Thermococcales and *Aeropyrum* of the order Desulfurococcales had been adapted to thrive in marine hydrothermal ecosystems and are able to grow at maximum temperature up to 100°C and above (Fiala and Stetter, 1986; Sako *et al*., 1996; Atomi *et al*., 2004). *Thermococcus kodakaraensis* KOD1 was found containing three subtilases: TK-subtilisin, alkaline serine protease (TK-SP) and subtilisin-like Tk-0076 (Tanaka *et al*., 2007; Foophow *et al*., 2010). The Tk-subtilisin and alkaline serine protease exhibited optimum enzymatic activity at 90°C and 100°C and half-lives at 100°C of 50 min and 100 min, respectively. Pyrolysin from *Pyrococcus furiosus* had a half-life value of 4 h at the boiling point of water (Voorhorst *et al*., 1996; Dai *et al.*, 2012). An aeropyrolysin (a metalloproteinase) from *Aeropyrum pernix* K1 (an aerobic marine hyperthermophilic archaeon) had a maximum activity at 110°C. The enzyme was extremely thermostable with half-lives of 2.5 h at 120°C and 1.2 h at 125°C, as well as displayed highly resistant to various denaturing reagents: urea, guanidine-HCl, dithiothreitol, 2-mercaptoethanol and SDS (Sako *et al*., 1997).

Phylogenetic Tree of Subtilases

Subtilases are often referred to as subtilisin-like serine proteases, the second largest serine protease family characterized to date and were classified based on similarity of amino acid sequences into 6 clans: subtilisins, thermitases, proteinases K, lantibiotic peptidase, pyrolysin and kesins (Siezen and Leunissen, 1997). Native extracellular and multimeric membrane bound subtilisin-like serine proteases referred to as fervidolysin (73 kDa) and islandisin (>200 kDa; subunit of 97 kDa) were purified from *Fervidobacterium pennivorans* and *F. islandicum*. The enzymes displayed their activities against keratin and casein. Three subtilisin-like serine proteases were reported in the complete genome sequences of *Fervidobacterium* (Nam *et al*., 2002; Kluskens *et al*., 2002; Kanoksilapatham *et al*., 2016). The accession numbers of the complete genome sequences and the protein identities are listed in Table 1. The amino acid sequences of the thermitase-like subtilase showed 80-90% similarity to other within the group, but only 31 and 34% similarity to the fervidolysin and islandisin, respectively. All the proteases from *Fervidobacterium* were classified in the thermitase clan together with the thermitases from *Thermoactinomyces* (SFX416858 and WP_083465054) (Fig. 1). On the other hand, the keratinase (KerA) from *Bacillus licheniformis* OWU 1411T (AAG00492) was grouped into the subtilisin clan. The subtilases from *Thermococcus kodakaraensis* KOD1 (Tk-subtilisin, Tk-SP-alkaline serine protease and subtilisin-like Tk-0076) were classified into three distinctive clans: Thermitase, proteinase K and pyrolysin, respectively (Fig. 1).

Table 1: Growth temperatures and protein identities of serine proteases reported in the complete genomes sequences of *Fervidobacterium* spp.

Microorganisms	Optimum growth temperature, range (°C)	Fervidolysin and fervidolysin-like serine protease	Islandisin and islandisin-like serine protease	Thermitase-like subtilase	Complete genome sequences, GenBank Nos.
F. pennivorans DSM 9078[T]	70, 50-80				NC_017095
Protein code		WP_014451857[c]	WP_014451869[d]	WP_014451703	
Length (aa)[a]		699	697	439	
Mol. mass (kDa)[b]		75	76	48	
F. islandicum AW-1	70, 40-80				NZ_CP014334
Protein code		WP_033191969	WP_052107197	WP_033191846	
Length (aa)[a]		699	701	439	
Mol. mass (kDa)b		76	76	48	
F. thailandense FC2004[T]	78-80, 60-88				LWAF0100000
Protein code		WP_069293857	WP_069292648	WP_069293332	
Length (aa)[a]		709	522	445	
Mol. mass (kDa)[b]		77	56	49	
F changbaicum DSM 17883	75-80, 55-90				FNDL0000000
Protein code		SDH01295	SDH01707	SDH53197	
Length (aa)[a]		703	701	439	
Mol. mass (kDa)[b]		76	76	48	
Protein code		SDH01361			
Length (aa)[a]		686			
Mol. mass (kDa)[b]		73			
F. nodosum Rt17-B1	70, 47-80				NC_009718
Protein code		WP_011994224	WP_011994230	WP_011993735	
Length (aa)[a]		710	682	440	
Mol. mass (kDa)[b]		76	75	48	
F. gondwanense DSM 13020	65-68, >45–<80				FRDJ00000000
Protein code		WP_072760880	WP_072760817	WP_072758935	
Length (aa)[b]		694	683	437	
Mol. mass (kDa)[b]		75	75	48	

[a]aa is abbreviation of amino acids
[b]Molecular mass includes the mass of signal peptide,
[c]Fervidolysin was first identified in *F. pennivorans* (Friedrich and Antranikian, 1996),
[d]Islandisin was first identified in *F. islandicum* AW-1(Nam *et al*., 2002)

Keratin and Biodegradation

Keratin

Keratin is insoluble in water and organic solvents thus it is extremely resistant to proteolysis by many proteases and difficult to degrade under natural conditions. However, it is naturally degradable by keratinolytic microorganisms. Two types of keratin differ slightly in structure, composition and properties: α- and β-keratins. Alpha-keratin is a fibrous protein that is rich in alpha helices and found as the primary component in hairs, horns, nails and the epidermis layer of the skin of mammals. Unlike α-keratin, β-keratin is rich in β pleated sheets and found in reptiles, as well as bird's scales, beaks, claws and feathers. The structure of β-keratin type accounted for much more rigidity to reptilian skin and avian feather than the α-keratin does to mammalian skin. Keratin hydrolysates can be found their applications in hair care products including shampoo, coloring spray, toner, etc., to improve strength and protect hair fibers and texture from chemical and environmental damages (Dias, 2015).

Biodegradation

Keratinous wastes are a major by-product of poultry, slaughterhouse, leather-processing and fur-processing industries. Due to the growing of poultry industries, feathers are the most common keratinous waste generated from poultry farms and slaughterhouses. Feathers comprise about 90% of protein and they are made up approximately 8% body weight of adult chicken. The increased production of poultry industry results in large amount of waste that required proper management (Brandelli *et al*., 2015). Feather meal is used in formulated animal feed and in organic fertilizer. However, its poor digestibility and insufficient essential amino acids composition are major limitations for direct utilization in animal feeds. Biodegradation employing either keratinolytic microorganisms or keratinases for pretreatment of feather waste were revealed significantly improved the digestibility of feather meal and the bacterial biomass improved the content of some essential amino acids (such as lysine, histidine and methionine) (Shih, 1993; Bertscha and Coello, 2005).

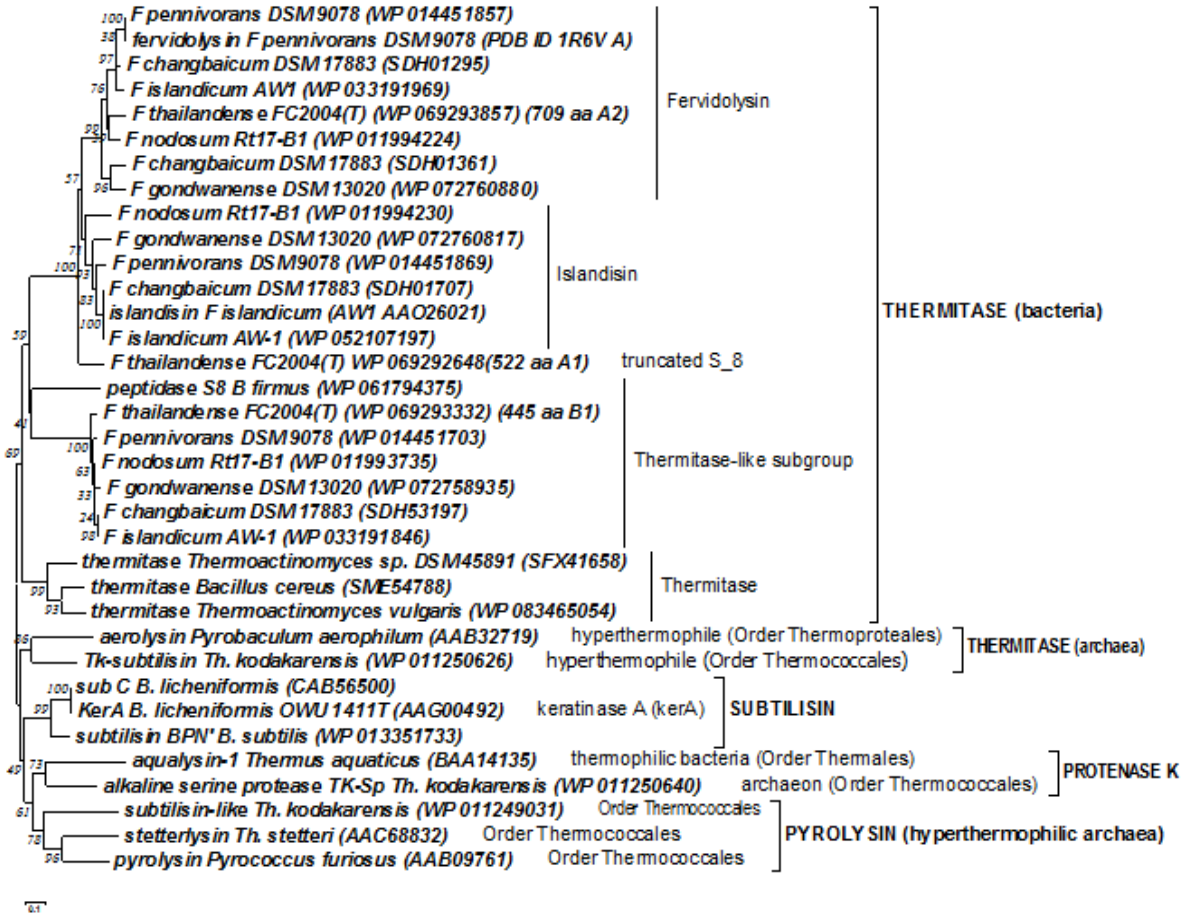

Fig. 1: Phylogenetic tree of subtiases family and keratinases from *Fervidobacterium* spp.. The tree was constructed using the MEGA6 software. Bootstrap values as a percentage of 1000 replications are presented. Bar indicates 0.1 changes per amino acid position. Clans of enzyme are shown following one-sided brackets. Subgroups of a clan are shown following vertical lines

A numbers of waste management systems have been employed for the treatment of poultry waste including incineration, chemical and/or biological treatments. Incineration process consume energy and generate large amount of carbon dioxide and the heat alkaline treatment destroy some essential amino acids and lead to formation of toxic non-nutritive amino acids. Biodegradation employing microorganisms has several advantages, such as safe process, cost effective and decreases in carbon dioxide generation. Unfortunately, biodegradation of feather employing mesophiles allows propagation of many human and animal pathogens including keratinolytic fungi that cause skin diseases. On the other hand, utilization of thermophiles and hyperthermophiles for the treatment of poultry waste at high temperature is safer from pathogens and toxin producing microorganisms. Particularly, when the feather meal by product is formulated for animal feeds, the risk of disease and toxin transmission to livestock be minimized (Wang and Parsons, 1997; Friedman, 1999; Suzuki *et al*., 2006; Korniłłowicz-Kowalska and Bohacz, 2011; Mézes and Tamás, 2015).

Various biodegradation systems have been designed for the treatment of feather waste with particular keratinolytic microorganism and/or enzymatic reaction. Some examples include a two-stage alkaline-enzymatic hydrolysis of feather in hot potassium hydroxide and 5% enzyme at 70°C, resulting in approx. 91% degradation (Mokrejs *et al*., 2011). An aerobic acidulocomposting was tested with moderate thermophile (*Meiothermus ruber*) in a bio-type garbage-treatment machine (BioClean BC-02). Approx. 70% of the chicken feathers (30 g) were digested within 14 days (Shigeri *et al*., 2009). Two-stage fermentation system employing keratinolytic, mesophilic *Bacillus licheniformis* KK1 and an anaerobic hyperthermophilic archaeon, *Thermococcus litoralis* was successfully tested for bio-hydrogen production from keratin-rich waste. In this specific case, *Bacillus licheniformis* KK1 was employed to convert keratin-containing waste into amino acids and peptides rich fermentation product. Then the keratin fermentation product supplemented with essential minerals was metabolized by *Thermococcus litoralis*, an anaerobic hyperthermophilic archaeon. *T.*

litoralis grew on the keratin hydrolysate and produced hydrogen gas as a physiological fermentation byproduct (Bálint *et al*., 2005).

Thermophilic and extremely thermophilic species of *Fervidobacterium* have been recognized as fast feather degraders: *F. pennivorans*, *F. islandicum* AW-1, *F. thailandense* FC2004 and *Fervidobacterium* sp. FA004. The strain FA004 were grown anaerobically in medium supplemented with 33 g/l of feather at 76°C and degraded white and black feathers differently with fascinating rates of 81% for white-chicken, 32% for black-chicken and 33% for grey-goose feathers within 6 days (Fig. 2). Although *Fervidobacterium* have been acceptable as top candidates for treatment of feather waste, biodegradation dealing with high temperature and strictly anaerobic condition may hinder the feasibility for scaling up.

Applications of Keratinases

Bio-Safety on Infectious Prion Proteins

Prions are infectious agents composed entirely of a protein material that are able to induce abnormal folding of normal proteins of infected patients. Prions can cause transmissible spongiform encephalopathies of brain and nervous system including bovine spongiform encephalopathy, scrapie and Creutzfeldt–Jakob disease. Some bacterial and archaeal keratinases hydrolyze not only the hard-degrading keratinous fiber but also digest the protease resistant aggregated prion proteins (Mitsuiki *et al*., 2006; Šnajder *et al*., 2012; Suzuki *et al*., 2006; Gupta *et al*., 2013). Keratinase from strain PWD-1, a serine protease of a moderate thermophile *Bacillus licheniformis* (Lin *et al*., 1992) was the first reported protease capable of degrading the prions. However, digestion of prion protein by PWD-1 requires pretreatment at 115°C for 40 min in the presence of SDS (Langeveld *et al*., 2003). Pernisine, an extremely thermostable serine metalloprotease produced by an aerobic marine archaeon, *Aeropyrum pernix* K1 digest pathological prion protein completely at 92°C. The purified pernisine was active between 58-99°C and between pH 3.5-8.0. The temperature and pH optima in the presence of $CaCl_2$ were about 105°C and pH 6.5 (Šnajder *et al*., 2012). Purified keratinase from the strain MSK103 was also capable of degrading and decontaminating prion infected brain homogenate at 50°C without detergents or heat pretreatment (Yoshioka *et al*., 2007). The MSK103 enzyme had strong activity between 60-70°C and optimal pH was 9-10. Alkaline serine protease from *Streptomyces* strain 99-GP-2D-5 degraded scrapie prion within 3min. The maximal activity of the enzyme was reported at 60°C and pH 11 (Hui *et al*., 2004).

Industries, Cosmetics and Pharmaceutical Products

Applications of thermoactive keratinase as ingredient in detergent have been successfully applied for removal of food stained-clothes in washing machine. Leather-processing industry usually employs sodium sulfide for de-hairing process step. However, sodium sulfide has unpleasant odor and can pollute environment. Due to keratinase is able to digest collagen in epidermis layers, hair can be easily rubbed off after treatment with enzyme. The de-hairing step employed a subtilisin-like keratinase instead of the toxic sodium sulfide is an environmental clean process (Macedo *et al*., 2005; Giongo *et al*., 2007).

Action of keratinase on nail and skin keratins facilitate removal of dead cells on the nail and skin surfaces. Topical drugs and various skin conditions have included keratinase to remove excessive keratin and thus improve efficacy of the treatment of hyperkeratosis (a thickening of skin resulted from accumulation of excess quantity of keratin) and acne. Keratinase in facial scrubs, anti-dandruff shampoo and other personal care products for hair removal and young looking skin are now on markets (Gupta *et al*., 2013).

Fig. 2: Degradation of feathers at 76°C by *Fervidobacterium* sp. strain FA004

Conclusion

This work highlighted profound information on keratin degrading microorganisms and applications, focusing on keratinolytic enzymes from thermophiles and hyperthermophiles. Keratin and keratinase have found their applications in various sectors. Novel property of keratinolytic enzymes in hydrolysis of infectious prion proteins has inspired a new research field. Thermophiles have a number of advantages over mesophiles. Biodegradation of feather waste dealing with thermophiles appear to be a candidate of choice. However, this process consumes energy.

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Author's Contributions

Weeranut Intagun: Conception and design, Acquisition of data, Drafting the article and Final approval of the version to be submitted and any revised version.

Wirojne Kanoksilapatham: Conception and design, Analysis and interpretation of data, Reviewing it critically for significant intellectual content. Final approval of the version to be submitted and any revised version.

Ethics

This review article is original and have never been published anywhere. The corresponding author confirms that all of the authors have read and approved the manuscript and no ethical issues involved.

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