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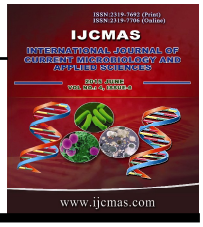

Review Article

Bacterial keratinases and their prospective applications: A review

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A B S T R A C T

Keratin, an insoluble fibrous structural protein is present in hair, feather, nails, wool and horns of animals which can be used as source of amino acids, animal feed and fertilizers. Keratin is highly stable with low degradation rate due to presence of disulfide bridges, hydrogen bonds and hydrophobic interactions. Keratin is degraded by ubiqitin proteasome pathway, mainly in eucaryotes. Keratinase, an extracellular enzyme, which degrade keratin in prokaryotes. Keratinases are produced by species of *Bacillus, Streptomyces, Aspergillus, Fervidobacterium, Xanthomonas, Chryseobacterium and Vibrio.* Keratinases are useful in enzymatic de-hairing in leather and cosmetic industry, also eliminates the use of sodium sulfide and bate in deliming, also provides hair of good quality. Amino acids produced from feather recycling by keratinases used as a nutrient rich source for *Thermococcus litoralis,* produces good amount of hydrogen as byproduct. Hydrolysis of pathogenic forms of prion protein by keratinases constitutes a novel outstanding application of this enzyme. The accumulation of PrP^{sc}, proteosome resistant prion protein, causes transmissible spongiform encephalopathies (TSEs), a progressive neurological disease. These pathogenic prion proteins are resistant to digestion by proteases but sensitive to keratinases.

Introduction

Keratin is the key structural material of outer layer of human skin. It is also the key component of hair, nails, horn and wool. It belongs to family of fibrous structural protein in which monomers assembled to form intermediate filament. Keratin has very high stability and low degradation rate due to higher degree of disulfide bridges, hydrogen bond and hydrophobic interactions (Bradbury 1973). Keratin chain is tightly packed into α helix and β sheet into a super coiled polypeptide chain, resulting in mechanical stability (Fig. 1) (Kreplak *et al.* 2004). In addition to hydrogen bond present in chains, cysteine bridges confer higher degree of mechanical stability and resistance to proteolytic degradation. According to sulfur content, keratin is grouped into two

families: one is hard keratin which includes feather, hair, nail, horn and hoof and other one is soft keratin which includes skin. In feather keratin, 7.3 residues of cysteine are present per 100 residues which provide it high mechanical strength (Table 1).

Keratin degradation in eucaryotes

Proteins are continually being hydrolyzed to their constituent amino acids by highly selective proteolytic systems. Interestingly, proteins are degraded at widely differing rates that can vary from minutes to days or weeks or months. This process of continually destroying cellular proteins has important homeostatic functions, such as regulating cell cycle, signal transduction, differentiation, and response to stress. In all tissues, the majority of intracellular proteins, one example of which is keratin, are degraded by the ubiquitin (Ub)-proteasome pathway (UPP). If the UPP fails to degrade intracellular proteins, the accumulation of misfolded protein may overload the proteasome, potentially leading to pathogenesis.

The degradation of a protein via the UPP involves two successive steps: (1) tagging of the substrate by covalent attachment of multiple Ub molecules, and (2) degradation of the tagged protein into small peptides by the 26S proteasome complex with release of free and reusable Ub (Fig. 1). The 2004 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose for the discovery of the UPP.

Ubiquitin is a small regulatory protein that has been found in almost all tissues of eukaryotic organisms. It directs proteins to compartments in the cell, including the proteasome which destroys and recycles proteins.. A key glycine residue within the C-terminus is required for its conjugation to other Ub molecules and target substrates; it also contains internal lysine residues that are required for the formation of polyubiquitin chains.

The degradation of keratin by Ub involves mainly four steps (Fig 2) (Rogel *et al.* 2010):

1. Activation of Ub by Ub activating enzyme E1. This enzyme uses ATP to generate Ub thioester, a highly reactive form of ubiqitin (Pickart 2001; Ciechanover and Schwartz 1998).
2. Once activated, Ub bound to E1 is now transferred to sulfhydryl group of one of Ub carrier protein or E2. The E2s are small protein that shares a conserved 16-kD core containing cysteine that forms a thioester linkage with the activated Ub.
3. E3 are ligases, function either as single protein or in complexes (Ciechanover and Schwartz 1998). E3 act as scaffolds and catalyze the transfer of the activated Ub from E2s to a lysine in the target protein and subsequently to lysines that are present in Ub, yielding a substrate anchored chain of Ub molecule.
4. 26S proteasome, found in both cytosol and nucleus of cell, catalyzes the rapid degradation of ubiquitinated proteins (Goldberg 2003). This enormous complex is composed of central barrel shaped 20S core particle with 19S regulatory particle at either or both of its ends (Groll *et al.* 1997). The proteolytic activity of 26S proteasome lies within the 26S core. It is composed of four stacked, hollow rings, each containing seven distinct but related subunits (Lowe *et al.* 1995). The outer α rings are identical, as are the two inner β rings. The outer α subunit of 20S particle surrounds a narrow, central and gated pore through which substrate s enters and product exit (Lowe *et al.* 1995). The physical

architecture of the 19S particle selects, prepares, and translocates substrate into the 20S core for degradation. The outer lid of the 19S particle contains subunits that bind polyubiqitin chains, as well as two de-ubiquitinating enzymes that dissemble Ub chains, allowing for reuse of Ub in the degradation of other proteins (Glickman *et al.* 1998). After de-ubiqitination, target proteins are unfolded and processed through the 20S particle in an ATP dependent manner. After the substrate enters the central chamber of the 20S particle, the polypeptide is cleaved by the six proteolytic sites on the inner face of the chamber, resulting in small peptides that range from 3 to 23 residues in length (Kisselev *et al.* 1999). These peptides are then rapidly digested into constituent amino acids by the abundant cytosolic endopeptidases and amino peptidases and reused to synthesize new proteins or are metabolized (Tamura *et al.* 1998).

Keratin degradation in procaryotes by keratinases

Keratinases are proteolytic enzymes in nature. It was classified as proteinase with EC number 3.4.11.25 (Riffel and Brandelli 2006).

Keratinases are produced only in the presence of keratin containing substrate. It mainly attacks on the disulfide (-S-S-) bond of the keratin substrate (Bockel *et al.* 1995).

Sources of keratinases

Keratinolytic enzymes are widespread in nature and are produced by a group of microorganisms largely isolated from poultry wastes. A vast variety of bacteria, actinomycetes and fungi are known to be keratin degraders. The important degraders are listed in Table 2. Among bacteria, degradation is mostly confined to gram-positive, including *Baciilus licheniformis, Bacillus subtilis, B pseudofirmus and Microbacterium* sp kr 10. However, a few strains of gram-negative bacteria, viz. *Vibrio, Xanthomonas,* and *Chryseobacterium* (Sangali and Brandelli 2000; De Toni *et al.* 2002; Lucas *et al.* 2003), have also been recently reported. In addition, a few thermophiles and extremophiles belonging to the genera *Fervidobacterium pennavorans, Thermoanaerobacter* and *Bacillus* have also been described (Rissen and Antranikian 2001; Nam *et al.* 2002; Gassesse *et al.* 2003). Besides these, actinomycetes from the Streptomyces group, viz. *S. fradiae* (Novel and Nickerson 1959), *S. pactum* (Bockle *et al.* 1995), *S. thermoviolaceus* SD8 (Chitte *et al.* 1999) and the Thermoactinomyces group, viz. *T. candidus* (Riffel and Brandelli 2006), is commonly described as keratin degraders. The most keratinolytic group among fungi belongs to fungi imperfectii including the following genera: *Chrysosporium, Aspergillus, Alternaria, Fusarium, Paecilomyces, Penicillium and Doratomyces*. However, they do not have much commercial value as most of them are categorized as dermatophytes (Gradisar *et al.* 2000).

Most of the keratinases are serine protease (Lin *et al.* 1992; Bockle *et al.* 1995; Suh and Lee 2001; Nam *et al.* 2002; Kojima *et al.* 2006) and very few are metalloprotease (Brouta *et al.* 2001; Allpress *et al.* 2002; Farag and Hasan 2004) (Table 2).

Serine proteases (or **serine endopeptidases**) are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the (enzyme's) active site. They are found ubiquitously in

both eukaryotes and prokaryotes. Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. Chymotrypsin-like serine proteases are characterised by a distinctive structure, consisting of two beta-barrel domains that converge at the catalytic active site. Subtilisin is a serine protease in prokaryotes. Subtilisin is evolutionarily unrelated to the chymotrypsin-clan, but shares the same catalytic mechanism utilising a catalytic triad, to create a nucleophilic serine. Keratinase from *B licheniformis* PWD 1 is well characterized serine protease, having N terminal homology with Carlsberg subtilisin (Lin *et al.* 1992) (Table 3). It is coded by kerA, is expressed specifically for feather hydrolysis (Lin *et al.* 1995). Keratinolytic enzymes from species of *Bacillus* strongly inhibited by PMSF (polymethylsulfonyl flouride), benzamidine or EDTA, indicating that keratinases belong to serine protease family.

Serine protease mechanism: *Ping Pong Catalysis*

The triad is a coordinated structure consisting of three essential amino acids: histidine (His 168), serine (Ser 325) (hence the name "serine protease") and aspartic acid (Asp 137). The catalysis of the peptide cleavage can be seen as a ping-pong catalysis, in which a substrate binds (in this case, the polypeptide being cleaved), a product is released (the N-terminus "half" of the peptide), another substrate binds (in this case, water), and another product is released (the C-terminus "half" of the peptide).

Each amino acid in the triad performs a specific task in this process (Fig 3):

- The serine has an -OH group that is able to act as a nucleophile, attacking the carbonyl carbon of the scissile peptide bond of the substrate. The polypeptide substrate binds to the surface of the serine protease enzyme such that the scissile bond is inserted into the active site of the enzyme, with the carbonyl carbon of this bond positioned near the nucleophilic serine.
- The serine -OH attacks the carbonyl carbon, and the nitrogen of the histidine accepts the hydrogen from the -OH of the [serine] and a pair of electrons from the double bond of the carbonyl oxygen moves to the oxygen. As a result, a tetrahedral intermediate is generated.
- The bond joining the nitrogen and the carbon in the peptide bond is now broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move back from the negative oxygen to recreate the bond, generating an acyl-enzyme intermediate.
- Now, water comes in to the reaction. Water replaces the N-terminus of the cleaved peptide, and attacks the carbonyl carbon. Once again, the electrons from the double bond move to the oxygen making it negative, as the bond between the oxygen of the water and the carbon is formed. This is coordinated by the nitrogen of the histidine, which accepts a proton from the water. Overall, this generates another tetrahedral intermediate.
- In a final reaction, the bond formed in the first step between the serine and the carbonyl carbon moves to attack the hydrogen that the histidine just acquired. The now electron-deficient carbonyl carbon re-forms the double bond with the oxygen. As a result, the C-terminus of the peptide is now ejected.

Applications of keratinases

Use of keratinases arose an important alternative for recycling of keratinous byproducts, particularly from poultry and leather industry. The development of bioprocesses that can convert the huge amount of such byproducts into value added products have been investigated and found that keratinase have the ability to hydrolyze diverse substrates indicating the potential of such enzymes for bio-conversion of waste to value added products.

Bio-processing of poultry (keratin rich) waste:

Keratin is the main component of feather and represents 90% of feather weight, which constitute up to 10% of total chicken weight (Harrap and Woods 1964; Cherry *et al.* 1975). The increased amount of feathers generated by commercial poultry processing may represent a pollutant problem and needs adequate management (Shih 1993). Currently feathers are converted into feather meal by steam pressure cooking which require high energy input. Feather meal has been used in animal feed on limited basis as an ingredient, as it is deficient in histidine, methionine and tryptophan (Papadopoulos *et al.* 1986; Wang and Parsons 1997). The alternative option to feather meal by steam pressure cooking is enzymatic hydrolysis of feather by keratinases (Onifade *et al.* 1998; Grazziotin *et al.* 2006). Comparable growth rate was observed between chickens fed with soyabean meal and those fed with feather meal fermented with *Streptomyces* sp and *Bacillus* sp plus methionine supplementation (Elmayergi and Smith 1971). Crude keratinase from *B licheniformis* significantly increased total amino acid digestibility and is commercially available under the name of versazyme (Shih and Williams 1990; Lee *et al.* 1991; Brutt and Ichida 1999).

Grazziotin *et al.*(2006) conducted a study which involves production of enzymatic hydrolysates from chicken feathers and thus evaluating their in vitro digestibility. Feather hydrolysate was produced by using whole culture on 60 g/l (WCH) or only culture supernatant of cultivation on 10 g/l (CSH). Amino acid composition of hydrolysate determined indicating in methionine, lysine and histidine.

Culture supernatant hydrolysate showed high amount of sulphur containing amnio acid. Whole culture hydrolysate was found to be rich in serine, leucine and glutamate while culture supernatant hydrolysate was rich in serine, arginine and glutamate. Feather meal had lower amount of essential amino acid in comparison to CSH & WCH (Table 4).

In vitro digestion by pepsin and pancreatic enzyme was determined. The controls (casein and soybean protein) showed similar digestibility. WCH had lower digestibility than controls but higher than feather meal and milled feather. CSH showed similar digestibility as that of controls but have relatively low protein content (Table 5).

Leather bio-processing

Leather and environment can be described as two sides of the coin. Leather production yields significant quantities of organic waste, a significant portion of which originate from keratin. Approximately 35-40 liter of water is used per kg of hide processed. With the present annual global processing of 9 X 10^9 liter hides and skin, it is estimated that 30-40 X 10^{10} liters of liquid effluent is generated. This gives rise to three major problems i.e. availability of good water, treatment of effluent and sulfide emissions during de-hairing. In terms of BOD, COD and total dissolved solids, almost 70% of pollution originates from pre-

tanning process (Ramasami *et al.* 1998; Ramasami *et al.* 1999; Marsal 1999). Leather processing mainly involves three steps:

1. Beam house or pre-tanning process: clean hides or skins
2. Tanning: permanently stabilize skins or hides
3. Post tanning and finishing: aesthetic value added

At each stage, various chemicals are used and variety of materials are expelled (Fig 4).

Sulfide is toxic and is major chemical used for de-hairing (Rao *et al.* 1997). It is now possible to reduce sulfide at its source using enzyme assisted processes. Enzymatic de-hairing generally uses proteolytic enzymes along with small amounts of sulfide and lime. Gehring (2002) reported that *Streptomyces griseus* and carbonate buffer 7 surfactant used to de-hair bovine hides. Similarly Thanikaivelan *et al.* (2004) too reported that 0.5% sodium sulfide and 1% enzyme concentration used for complete hair removal in cow skin. Enzyme assisted de-hairing reduces sulfide concentration to 85%. Reductions in effluent loads of COD and total solids for leather processing are 45% and 20%, respectively, compared with conventional leather processing. Total dry sludge is reduced from 152 kg to 12 kg per 1000 kg of raw hide processed (Thanikaivelan *et al.* 2002). This is one of the most pioneering achievements in context of solid waste management and total solid reduction.

Bio-hydrogen production:

There is increasing interest in utilization of renewable sources to satisfy the exponentially growing energy needs of mankind. Research on biological hydrogen production is propelled by the possible use of bio-hydrogen as the cleanest energy carrier and raw material (Benemann 1996). Bio-hydrogen is a part of a broader concept of developing zero emission technologies employing production of H_2 from biomass in photo-biological or heterotrophic fermentation routes (Cammack *et al.* 2001). Both processes depend on the supply of organic substrates and could be therefore ideally suited for coupling energy production with treatment of organic waste.

Balint *et al.* (2005) conducted a two step dark fermentation process joining together the keratin degradation ability of isolated *Bacillus* strain with the hydrogen production capabilities of an anaerobic archeon, *Thermococcus litoralis* (Fig 5). They isolated a novel aerobic *Bacillus* strain (*B licheniformis* KK1) which have outstanding keratinolytic activity. Then this isolated strain was employed to convert keratin containing bio-waste into a fermentation product that is rich in amino acids and peptides. The process was optimized for the second fermentation step, in which the product of keratin fermentation, supplemented with essential minerals was metabolized by *Thermococcus litoralis*, an anaerobic hyperthermophilic archeon. They observed a noticeable growth of *T litoralis* along with hydrogen production. Hydrogen production of *T litoralis* was routinely followed in 20 ml cuture and the effect of keratinolytic pretreatment was first studied. Using keratin hydrolyzed for at least 42 h resulted in a hydrogen concentration of approx. 50-70 ml H_2/l in the headspace of the thermophilic culture within 48 h. a hydrolysis time of less than 42 h led to inferior H_2 production, while significantly longer proteolysis time (92 and 138 h) did not greatly affect the H_2 production capacity. Therefore a keratin hydrolysis time of around 60 h is recommended (Table 6).

Table.1 Amino acid composition of feather keratin (Source: Riffel and Brandelli 2006)

Amino acid	# residues/ per 100 residues	Nature of amino acid
Alanine	4.2	Hydrophobic
Arginine	5.2	Hydrophilic
Asparagine	3.1	Hydrophilic
Aspartic acid	2.1	Hydrophilic
Cysteine	7.3	Hydrophobic
Glycine	11.5	Hydrophobic
Glutamine	5.2	Hydrophilic
Isoleucine	5.2	Hydrophobic
Leucine	6.3	Hydrophobic
Phenylalanine	4.2	Hydrophobic
Proline	11.5	Hydrophobic
Serine	16.7	Hydrophilic

Table.2 Biochemical properties of some of keratinolytic bacteria

Gram nature	Producer bacteria	Catalytic type	Reference
Gram positive	*Baciilus licheniformis* PWD 1	Serine	Lin *et al.* 1992
	B subtilis KS 1	Serine	Suh & Lee 2001
	B pseudofirmus FA 30-10	Serine	Kojima *et al.* 2006
	Streptomyces pactum DSM 40530	Serine	Bockle *et al.* 1995
	Fervidobacterium pennavorans	Serine	Freidrich & Antranikian 1996
	Microbacterium sp kr 10	Metallo	Thys & Brandelli 2006
Gram negative	*Vibrio* sp	Serine	Sangali & Brandelli 2000
	Chryseobacterium sp kr 6	Metallo	Riffel *et al.* 2007

Table.3 N terminal sequence of some keratinases and similar protease

Enzyme	N terminal sequence	Reference
Carlsberg subtilisin	AQTVPYGIPLIKADK	Jacobs *et al.* 1985
Keratinase B licheniformis PWD 1	AQTVPYGIPLIKADK	Lin *et al.* 1995
Subtilisin E	AQSVPYGISQIKAPA	Stahl & Ferrari 1984
Keratinase *B subtilis*	AQSVPYGISQIKAPA	Macedo *et al.* 2005
Keratinase *B pseudofirmus*	XQTVPXGIPYIYSDD	Kojima *et al.* 2006

Table.4 Amino acid composition of feather meal and feather hydrolysate (mg amino acid/g CP)

Amino acid	CSH	WCH	Feather meal
Ala	54.2	53.4	40.1
Arg	84.3	79.6	67.5
Asp	57.8	61.4	58.3
Cys	65.1	55.5	48.3
Glu	92.2	117.5	96.1
Gly	59.6	71.0	66.5
His	9.3	7.7	6.5

(Source: Grazziotin et al. 2006)

Table.5 In vitro digestibility of protein (Source: Grazziotin et al. 2006)

Sample	Protein (mg/g)	Digestibility
Casein	811	0.994
Soybean protein	794	0.995
Milled feathers	905	0.096
Feather meal	807	0.578
WCH	903	0.834
CSH	718	0.985

Table.6 Overall hydrogen production (Source: Balint et al. 2005)

Degradation time (hours)	H_2 yield (ml/ g feather)
24	16
42	21
60	24
92	19
138	19

Degradation step was done in fermentor, while hydrogen evolution was carried out in hypovials

Fig.1 Secondary structure of keratin

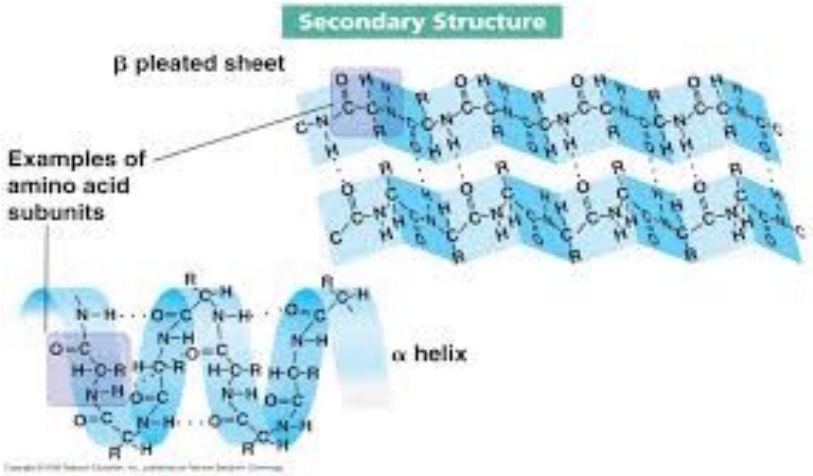
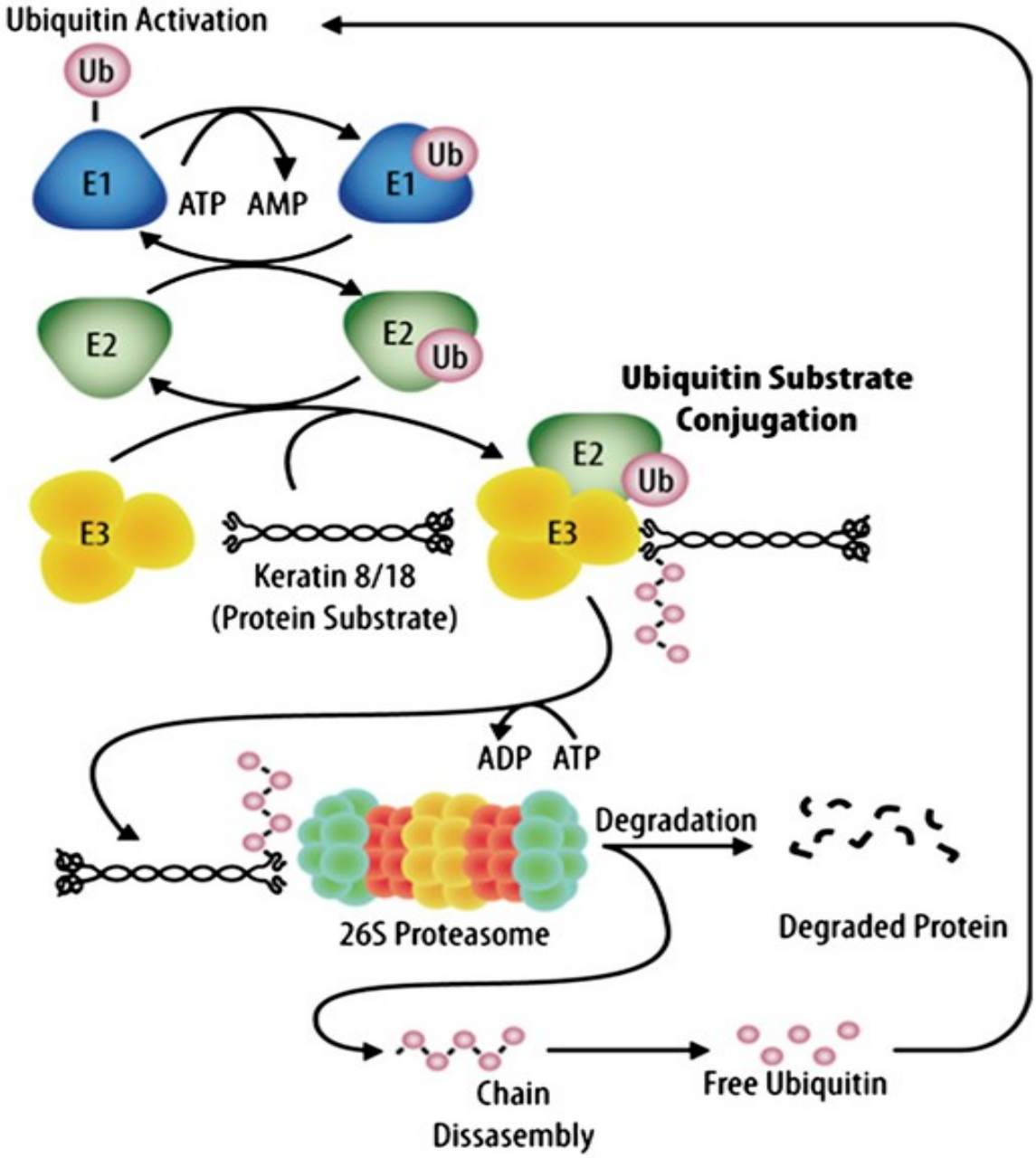
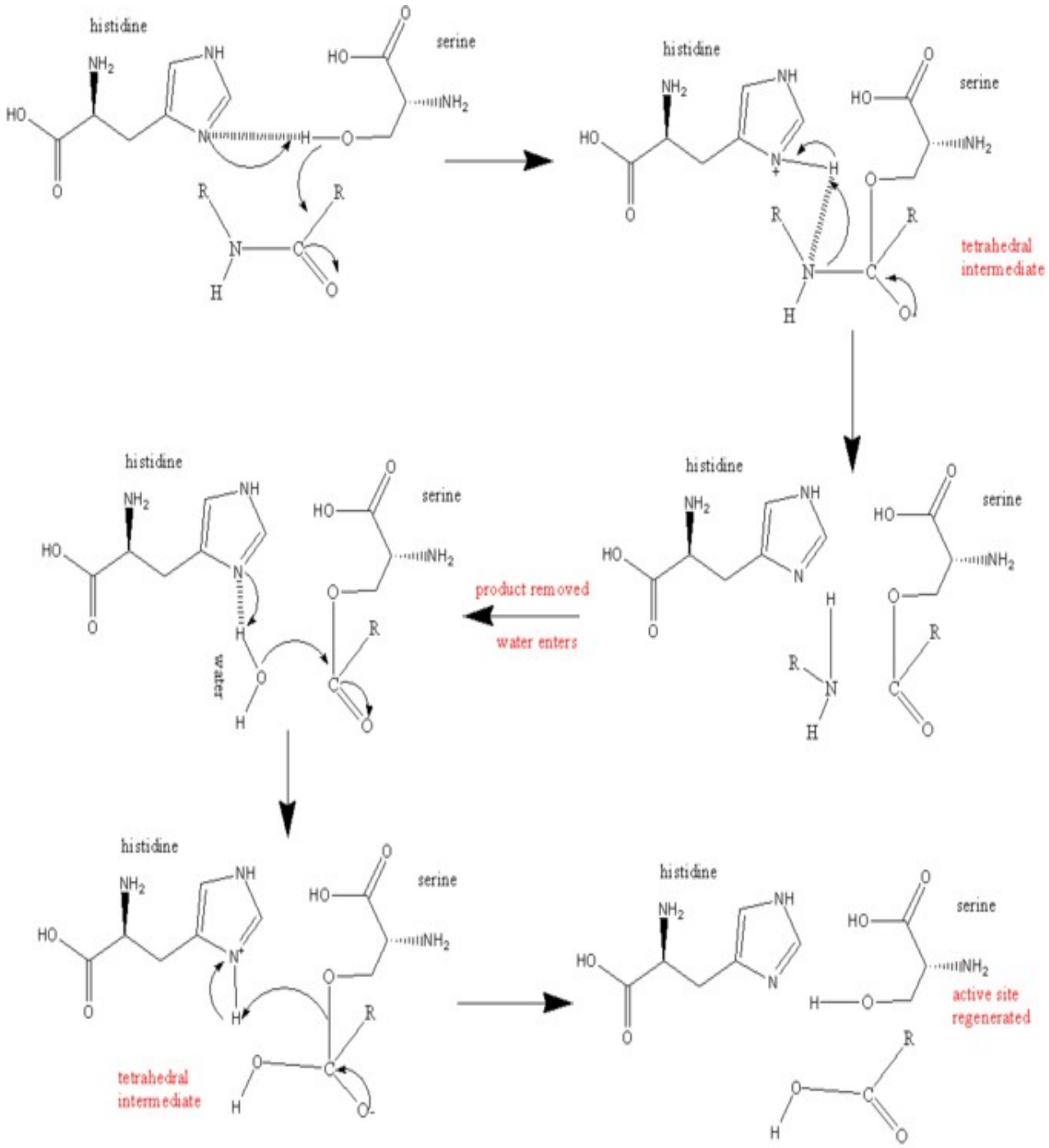


Fig.2 Keratin degradation via ubiquitin proteasome pathway (UPP)



Activation of Ub by Ub activating enzyme E1 to generate Ub thioester. Once activated, Ub now transferred to sulfhydryl group of one of Ub carrier protein or E2 that forms a thioester linkage with the activated Ub. E3 are ligases, function either as single protein or in complexes. E3 act as scaffolds and catalyze the transfer of the activated Ub from E2s to a lysine in the target protein and subsequently to lysines that are present in Ub, yielding a substrate anchored chain of Ub molecule. 26S proteasome complex is composed of central barrel shaped 20S core particle with 19S regulatory particle at either or both of its ends. The outer lid of the 19S particle contains subunits that bind polyubiqitin chains, as well as two de-ubiquitinating enzymes that dissemble Ub chains, allowing for reuse of Ub in the degradation of other proteins. After de-ubiqitination, target proteins are unfolded and processed through the 20S particle in an ATP dependent manner. After the substrate enters the central chamber of the 20S particle, the polypeptide is cleaved by the six proteolytic sites, resulting in small peptides that range from 3 to 23 residues in length (Source: Rogel *et al.* 2010).

Fig.3 Serine protease mechanism: Ping Pong catalysis



The serine has an -OH group that is able to act as a nucleophile, attacking the carbonyl carbon of the scissile peptide bond of the substrate. A pair of electrons on the histidine nitrogen has the ability to accept the hydrogen from the serine -OH group, thus coordinating the attack of the peptide bond. The carboxyl group on the aspartic acid in turn hydrogen bonds with the histidine, making the nitrogen atom mentioned above much more electronegative.

Fig.4 Inflow outflow diagram of leather processing (Source: Gehring et al. 2002)

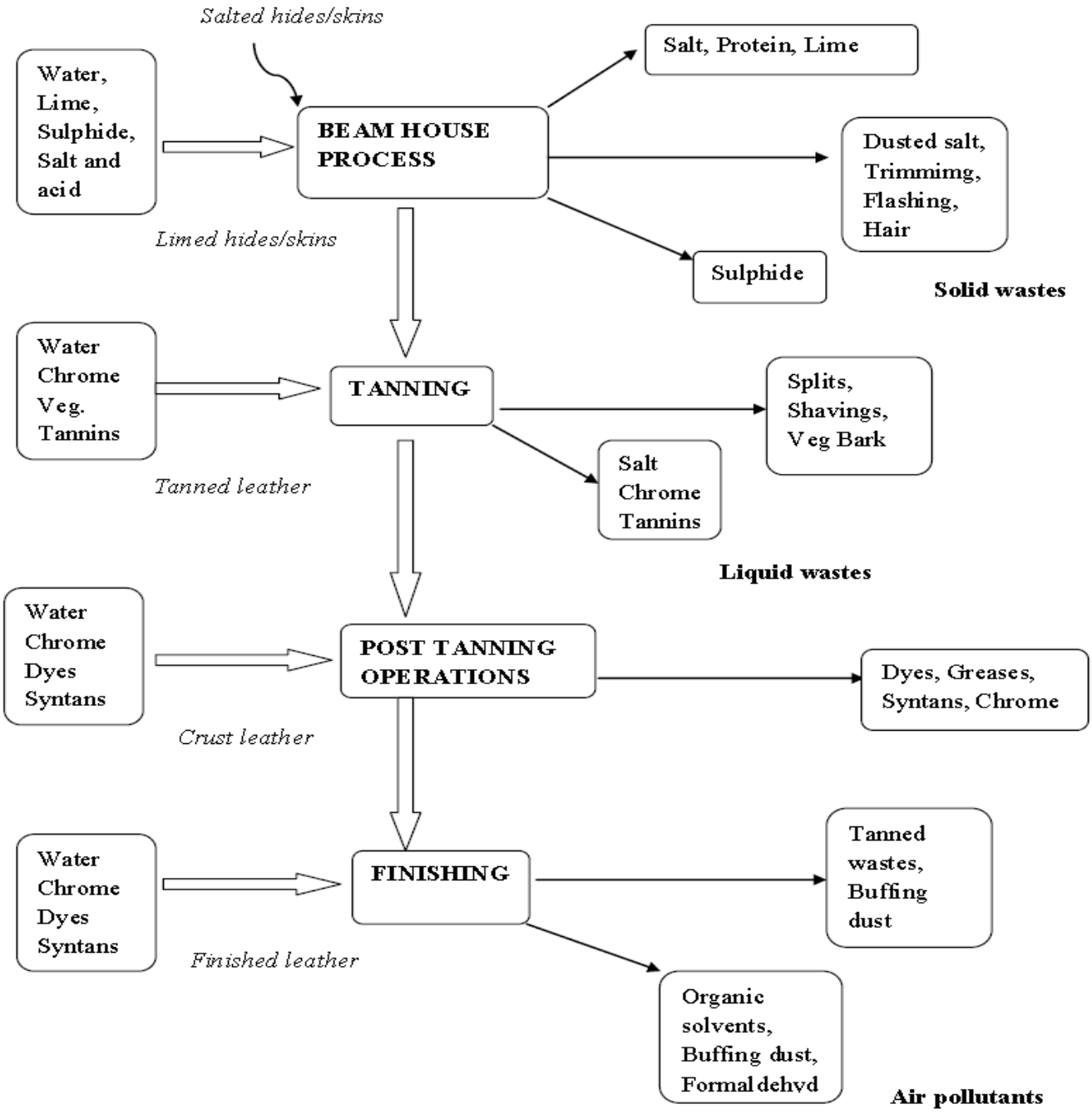
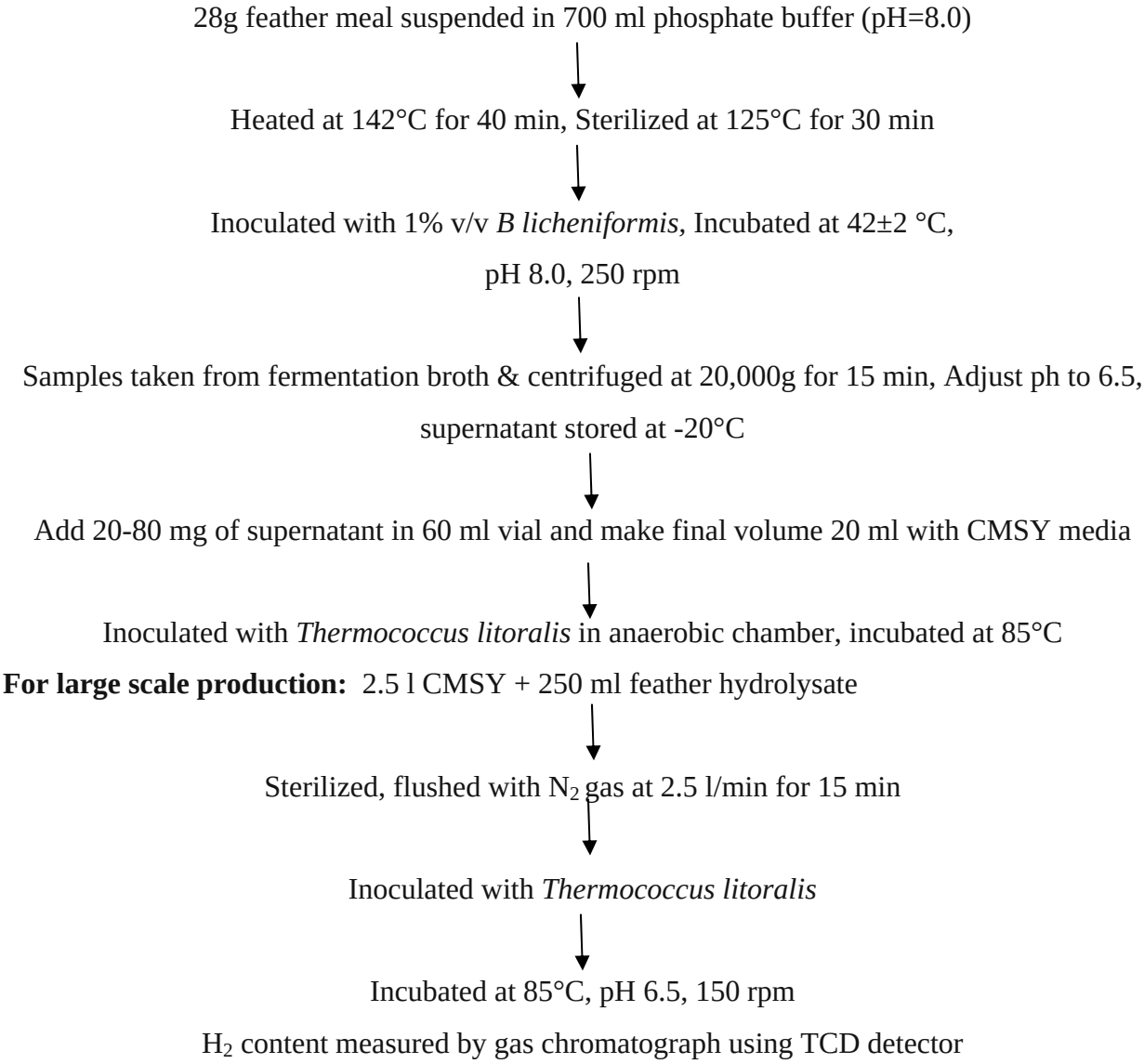


Fig.5 Flow chart of bio-hydrogen production from keratinous waste (Source: Balint et al. 2005)



The results indicate that maximal keratin degradation does not seem to be optimal for hydrogen production.

Degradation of pathogenic prion protein:

Prion is an infectious agent composed of protein in misfolded form. The word derived from two words i.e. protein and infection. The major prion protein is PrP (proteasome resistant protein). The expression of this protein is predominant in nervous system. The protein can fold into two ways i.e. exist in two forms:

1. PrP^c (normal cellular form): having 43% alpha helical and 3% beta sheet content. It is present in both pre and post synaptic neuron cells, but greatest concentration in pre-synaptic cells. The exact function of PrP^c is yet unknown but possibly involved in transport of ionic copper to cells from surrounding.

2. PrP^{sc} (disease causing/scrapie form): having 30% alpha helix and 43% beta sheet. That's why it is extremely resistant to proteolysis (Caughey *et al.* 1991; Pan *et al.* 1993). Its accumulation is a pathological cause of neurodegeneration. It causes disease called transmissible spongiform encephalopathies (TSEs), bovine spongiform encephalopathies (BSE) or mad cow, cruetzfeldt jakob disease in human (Chesebro 1990).

Yoshioka *et al.* (2007) conducted an experiment to study the efficacy of keratinase produced by *B licheniformis* in degrading PrP^{sc} in infected brain homogenate from mice and cattle. They found that keratinase from *B licheniformis*PWD1 is capable of degrading PrP^{sc} in bovine spongiform encephalopathy (BSE) infected brain homogenate but degradation requires pretreatment at 115 °C for 40 min in presence of surfactant. Keratinase from *B licheniformis* PWD 1 is the first protease capable of degrading PrP^{sc} (Lin *et al* 1997).

Keratinases are valuable enzymes for bio-processing of keratinous waste. Pollution problems posed by poultry waste reduced by use of keratinases. Their ability to degrade recalcitrant protein such as PrP^{sc}, constitute a remarkable property. Increased information on keratinolytic micro-organisms and the biochemical properties of their keratinases became available, allowing a better understanding of the biological waste.

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