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Biotechnological Aspects of Production, Characterization and Application of Keratinase

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ABSTRACT

Keratin is insoluble biopolymer and mainly present in tissues of birds, amphibians, reptiles and mammals. Many industries use keratin as raw material and accumulation of keratin causes many environmental problems. To reduce keratin accumulation there should be a need to develop an easy and cheap process for environmental remediation of keratin. Microbial keratinase, a proteolytic enzyme, is used to reduce pollution and is mainly obtained from Keratinophilic fungi, bacteria and actinomyces and Streptomyces. Keratin is degraded in prokaryotes by degrading serine protease and in eukaryotes by ubiquitin (Ub)-proteasome pathway (UPP). Keratinase used to degrade feathers of poultry and converted into high energy input feather meal, in leather industry for management of wastes which mainly causes the water pollution, by producing bio hydrogen from solid wastes. In this review it is investigated that microbial keratinase is less expensive than conventional production of keratinase and have many advantages to reduce pollution.

Keywords : Keratinophilic fungi, Streptomyces, actinomyces, serine protease, ubiquitin proteasome pathway, bio-hydrogen.

I. INTRODUCTION

Keratin is insoluble biopolymer due to the presence of sclera protein which imparts hardness and fibrous characteristics and become unreactive against chemicals. It is mainly present in tissues of reptiles, birds, amphibians, and mammals (Martınez and Velasco, 2012). It is used to prevent the loss of body fluids via making an outer coat of human and animal organs. Structural component of feathers, hair, nails, horns, hooves, bones, furs, claws, hides, bird beaks, skin, wool, scales, and bristle is made of keratin. Keratin is insoluble because it is rich in sulfur compounds with disulfide bridges, hydrogen bonds and hydrophobic interactions. It has a variety of amino acids, mainly cystine, lysine, proline, and serine. Keratins contain sclera protein which imparts hardness and become unreactive against most chemicals. It is not digested by pepsin, trypsin, or papain.it is (Kanchana, 2012). There are two types of keratin.α-helical coils of α-Keratin after self-assemblage converted into intermediate filaments (McKittrick *et al*., 2012; Meyers *et al*., 2008). β-Keratin which comprises β-pleated sheets is (Meyers et al. 2008) is made up of from supramolecular fibril bundles (Bodde *et al*., 2011). α-keratins (alpha- helix) are usually present in the hair, wool, horns, nails, claws, and hooves of mammals, whereas the harder β-keratin (beta- sheets) is found in bird feathers, beaks, and claws. Keratin is also present in the epithelial cell types of digestive organs (liver, pancreas, intestine, and gallbladder), which mainly consist of hepatocytes, hepato biliary ductal cells, oval cells, enterocytes of the small intestine, colon, and goblet cells. β-Keratin is more is more easily degraded by some keratinases than α-keratin because of the fibril and porosity structure and it has less disulfide bonds (Gupta and Ramnani 2006).

Major cause of environmental problems starts from industries in which keratin use as the raw material and keratin accumulate in the environment. Indeed, 90% of feathers are keratin and millions of kilograms of feathers from poultry farms are also discarded annually to the environment (Acda , 2010). To reduce keratin accumulation there should be a need to develop an easy and cheap process for environmental remediation of keratin. Strongly inhibition of Keratinolytic enzymes from species of Bacillus is mostly occurring by PMSF (polymethylsulfonyl flouride), benzamidine or EDTA

which indicates that keratinases belong to serine protease family.

A. Production of Keratinase

First time it is discover that bacteria can be used to degrade keratin isolated (Molyneux, 1959) then keratinase of the S8 protease family from *Bacillus licheniformis* strain were firstly purify and characterized (Lin et al., 1992). At an early stage of the keratinase research several more keratinases found which were to be subtilisin-like proteases, belonging to the serine proteases (S8 family), mainly from *Bacillus* sp. and *Streptomyces* sp. (Brandelli et al., 2010). At the beginning keratinases were thought to be as serine proteases. Microbial keratinase such as Keratinophilic fungi, bacteria, and actinomycetes naturally reside on keratin wastes. Keratinophilic and non-keratinophilic both have capability to produce keratinase but there is a great difference of rate of production b/w these two types of fungi and rate of production is high in Keratinophilic fungi than non-keratinophilic.

1. Keratinophilic Fungi

Proteolytic enzymes which are produce by keratinophilic fungi are capable of decomposing keratinic waste materials (Ganaie *et al*., 2010). Several keratinophilic fungi use keratin as their carbon and nitrogen sources and live as parasites on keratinous material. It multiplies by asexual manner by producing conidia. There are many keratinophilic fungi which have several taxa including hyphomycetes and (Kumar *et al*., 2013) this hyphomycetes consist of both dermatophytic (e.g., Microsporum species) and non-dermatophytic (e.g., *Chrysosporium* species and other genera) keratinophilic fungi (Gugnani *et al.,* 2000). By morphological features of fungal macro- and micro-conidia, molecular methods, and using DNA sequence analysis are usually used to identifiy the Keratinophilic species (Pakshir *et al*., 2013).

For sulphito lysis, Keratinophilic fungi produce sulfide, after which the proteolytic enzymes released by the fungi can easily cleave the keratin disulfide bonds of cysteine a major amino acid in keratinous materials are broken down., The products released during the degradation process are cysteine, S- sulphocysteine, cysteine acid, cysteine, and inorganic sulfate. The presence of these products in the culture media is the indication of the occurrence of true keratinophilic fungi. Nonkeratinophilic fungi do not produce this type of products during degradation. Keratinophilic fungi are mainly anthropophilic (human loving) or zoophilic (animal loving). Mostly keratin wastes are accumulated in the soils (geophilic) and for isolation of many keratinophilic fungi there is a need to prepare soil samples. Geophilic habitats including public beaches, agricultural areas, public parks, gardens, and elementary schools have been found to contain keratinophilic fungi are used to prepare Soil samples (Ramesh and Hilda 1998; Shadzi *et al.*, 2002; Anbu *et al*., 2004; Gugnani *et al*., 2012; Kachuei *et al.*, 2012). They reported that isolated keratinophilic fungi have obtained different countries including Egypt, Spain, Australia, Palestine, Kuwait, India, Iran, and Malaysia (Kumar *et al*., 2013). From soils common isolates of keratinophilic fungi include *Microsporum gypseum, M.canis,M.fulvum, M.nanum,Trichophyton terrestre, T.ajelloi, T.mentagrophytes, T.interdigitale, T.verrucosum, T.equinum, T.rubrum, T.interdig- itale,T.schoenleinii, T.simii,Chrysosporium keratinophilum, C. pannicola, C. tropicum,C. indicum, C. anum, C. lobatum, C. evolceanui, and C. indicum* (Shadzi *et al*., 2002).

2. Keratin Degrading Bacterial Isolates

Like fungi, there are many bacterial isolates which are capable of degrading keratin. There is greater demand of bacteria in industrial applications than fungi due to its faster growth. Fungi include easier colonization of fungal hyphae into the harder keratin in contrast to bacteria. The isolated bacterial strains which produce keratinase and degrade keratin are largely composed of Bacillus; it includes *B. subtilis* and *B. licheniformis* (Matikeviciene *et al*., 2009). There are many several studies have investigate that keratinase produced by several bacterial species (Korkmaz *et al.,* 2004; Hoq *et al.,* 2005; Joshi *et al*., 2007; Cai *et al*., 2008; Cortezi *et al*., 2008).Some are Gram-positive which produce keratinase are *Lysobacter, Nesterenkonia, Kocuria*, and Microbacterium, some are Gram-negative which are *Vibrio, Xanthomonas, Stenotrophomonas*, *Chryseo* bacterium, *Fervido* bacterium and Thermo an-aerobacter, can also degrade keratin (Gupta and Ramnani, 2006).

3. Keratinase production from Actinomyces and Streptomyces

Moreover, there were many actinomycetes, Streptomyces group which are capable of producing keratinase, namely, *S. fradiae, Streptomyces* species A11, *S. pactum, S. albidoflavus, S. thermoviolaceus SD8, and S. graminofaciens*, as well as Thermo actinomyces candidus (Korniłłowicz and Bohacz, 2011).

Table 1: production of Keratinases from different species

Fungi		Bacteria	
Species	Optimal condition (pH)		Optimal condition (pH)
Aspergillus oryzae	8	*Clostridium sporogenes*	8
Doratomyces Microsporum	8- 9	*Microbacterium arborescens*	7
Paecilomyces marquandii	8	*Fervidobacterium islandicum*	9
Trichophyton rubrum	8	*Kytococcus sedentarius*	7.0 – 7.5
Microsporum gypseum	8	*Stenotrophomonas maltophilia*	7.8
Scopulariopsis brevicaulis	8	*Kocuria rosea*	7.5
Myrothecium verrucaria	8.3	*Xanthomonas maltophilia*	8
Chrysosporium keratinophilum	9	*Streptomyces thermoviolaceus*	8
Trichoderma atroviride	8 – 9	Bacillus pumilus	10

B. Secretion, Isolation and Optimal growth conditions of microbial keratinases

Keratinases mainly obtained from fungi, actinomycetes, and bacteria (Kowalska and Bohacz, 2011). By secretion fungal keratinases can be easily obtained and over bacterial keratinases in some cases these were preferable, even though the fungi grow slower than bacteria. There is a need to select efficient keratinase producer from several keratin producing strains. There are many important things in the selection process during screening of microbial enzymes like selected enzymes should be less expensive, eco-friendly, and efficient in work. keratinophillic fungi have higher rate of production of keratinase than non Keratinophilic fungi therefore it is preferable for production of keratinase. To screen proteolytic enzymes there are several methods have been offered including keratin-baiting, plate screening, spectrophotometric methods, and sequence-based amplification. Feather-degrading bacteria using the16S rDNA sequence Jeevana Lakshmi et al. identified it (Lakshmi *et al*., 2013). From all methods, the plate-clearing assay is one of the preferable methods due to its visual results, less expensive and easier than others. For the initial screening and isolation of keratinolytic species the keratin-baiting method is mostly used because of its direct selection of Keratinophilic species on the substrate. In this method, any keratin source (hair and feather are mostly used) can be the bait (Ramesh and Hilda 1998; Anbu *et al*., 2004).

For cultivation of isolated microbes, there is a need of suitable artificial growth media under optimal conditions to attain excess production of keratinase. Due to the suitability Sabouraud's dextrose is commonly used media to grow keratinophilic fungi (Ramesh and Hilda, 1998; Anbu et al.2004; Mahmoudabadi and Zarrin, 2008). To degrade the keratin commonly keratinophilic fungi will take a longer time (in weeks). In this experiment they informed that to obtain Keratinophilic fungal growth 4-8 weeks required through hair-baiting technique (Gugnani *et al.*, 2012).According the study of Kumar *et al.* (2013) the next step after isolation 2 to 4 weeks of incubation are essential for growth of Keratinophilic fungi, while some other scientists according to them 4-5 weeks are necessary for growth (Mahmoudabadi and Zarrin, 2008). From all these experiments at the end it is concluded that for optimal growth occur at room temperature. It has also been stated that keratinophilic fungi are less efficient to degrade keratin as compare to non keratinophilic fungi. Like Keratinophilic fungi are able to degrade 40% of keratin after 8 weeks, which is less than half (<20%) of that amount can be degraded in the case of non keratinophilic fungi (Kunert, 2000). It has been reported that most keratinophilic microbes under neutral and alkaline pH flourish well and the range being 6 to 9 (Jain and Sharma, 2012). Most keratinophilic fungi are mesophiles while some are thermotolerant like *M. gypseum* and some species of *Chrysosporium* (Korniłłowicz and Bohacz, 2011).It has been reported that keratinase production by most bacteria, actinomycetes, and fungi is favorable b/w the temperature range of 28°C to 50°C, while its production by Thermo an aerobacter and Fervido bacterium species is favorable at 70°C (Friedricht and Antranikian 1996 ; Riessen and Antarnikian 2001; Nam *et al*., 2002). Optimal keratinase production occurs at 90°C by Chryso-sporium keratinophilum and its shelf- life is 30 minutes (Dozie *et al*., 1994), whereas the thermophile Fervido bacterium has an optimum temperature of 100°C and a half-life of 90 min (Nam *et al*., 2002).

C. Keratin degradation in eukaryotes

By using highly selective proteolytic systems, proteins are constantly being hydrolyzed to their constituent amino acids. Mostly degradation rate of proteins is different that can vary from minutes to days or even weeks or months. Continual destruction of cellular proteins has some important homeostatic functions, such as regulating cell cycle, signal transduction, differentiation, and response to stress. The majority of intracellular proteins in all tissues for example keratins are degraded using the ubiquitin (Ub)-proteasome pathway (UPP). If this UPP pathway fails to degrade intracellular proteins then there is accumulation of misfolded protein may increase level of proteasome and actually leading to pathogenesis. (Rogel *et al*., 2010).

Mechanism of degradation of a protein

There are two steps which mainly involve in the degradation of a protein via the UPP involve: Substrate is labeled by covalent attachment of multiple Ub molecules. 26S proteasome complex used for the degradation of the labeled protein into small peptides, involves the release of free and re-usable Ub as shown in Fig.1. A small regulatory protein has mostly found in all tissues of eukaryotic organisms. The main function of Ubiquitin it guides proteins to compartments in the cell, including the proteasome which destroys and recycles proteins. The degradation of keratin by Ub involves mainly four steps (Fig 1) (Rogel *et al*., 2010):1. Activation of Ub is achieved by Ub activating enzyme E1. To generate Ub thioester this enzyme uses ATP to produce a highly reactive form of ubiqitin (Ciechanover and Schwartz 1998; Pickart 2001). 2. After activation Ub, it bound to E1 and transferred to sulfhydryl group of one Ub carrier protein or E2. These small E2 proteins that shares a conserved 16- kD core contain cysteine that is used to forms a thioester linkage with the activated Ub. 3. E3 is ligases which function either as single protein or in complexes (Ciechanover and Schwartz, 1998). E3 act as scaffolds and used in the transfer of the activated Ub from E2s to a lysine which is present in the target protein and producing a substrate anchored chain of Ub molecule. 4. The rapid degradation of ubiquitinated proteins is usually catalyzed by 26S proteasome which is mostly found in both cytosol and nucleus of cell. (Goldberg, 2003). The proteolytic activity of 26S proteasome present within the 26S core. It is composed of four arranged hollow rings in which each ring consist of seven distinct but related subunits (Lowe et al., 1995). The outer rings are identical than two inner rings. The outer subunit of 20S particle consists of a pore which is narrow in shape and present in center. It acts as a gate for entry of substrates and exit of products (Lowe *et al*., 1995).

Selection preparation and translocation of substrate into the 20S core for degradation is usually done by 19S particle. Subunits that bind polyubiqitin chains are present in outer lid of the 19S particle as well as two de-ubiquitinating enzymes that dissemble Ub chains permitting for reuse of Ub in the degradation of other proteins (Glickman *et al*., 1998). Unfolding and processing of target proteins after de- ubiqitination is usually done through the 20S particle in an ATP dependent manner. When the substrate enters the central chamber of the 20S particle the cleavage of polypeptide is completed by the six proteolytic sites on the inner face of the chamber and producing small peptides that range from 3 to 23 residues in length (Kisselev *et al*., 1999). Now there is a complete rapidly digestion of peptides into constituent amino acids by the activity of some proteolytic enzymes cytosolic endopeptidases and amino peptidases and again it is used to produce new proteins or are metabolized (Tamura *et al*., 1998).

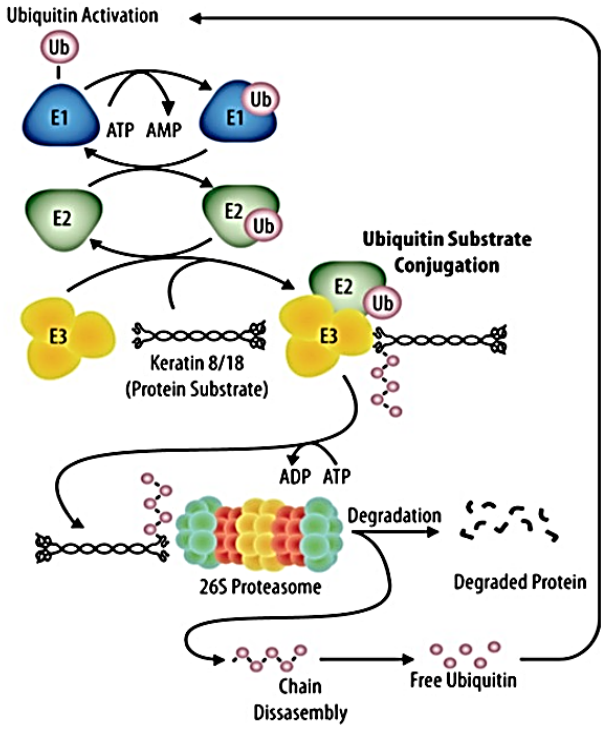

Figure 1 : Activation of Ub is achieved by Ub activating enzyme E1. To generate Ub thioester this enzyme uses

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D. Keratin degradation in prokaryotes by keratinases

Keratinases are proteolytic enzymes in nature and it mostly attack on disulfide (-S-S-) bond of the keratin substrate. In the presence of keratin containing substrate Keratinases are produced. Keratin degration in prokaryotes is usually done by degrading a serine protease which is Subtilisin (Bockel *et al.*, 1995; Riffel and Brandelli, 2006).Serine proteases are also called serine endopeptidases that are enzymes which are used to cleave peptide bonds in proteins. In this process serine serves as the nucleophilic amino acid at the active site of enzymes. They are found universally in both eukaryotes and prokaryotes. There are two broad categories serine proteases based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. Subtilisin is a serine protease of prokaryotes. From evolutionarily point of view subtilisin is distinct to the chymotrypsin-clan, but it is similar in the catalytic mechanism by utilizing a catalytic triad, to generate a nucleophilic serine (Harrap and Woods 1964; Cherry *et al*., 1975).

E. Applications of keratinases

For recycling of keratinous byproducts, particularly from poultry and leather industry keratinases is used as an important alternative. There is a development of bioprocesses that is used for bioconversion a large amount of byproducts into valuable products and there is a need to investigate either keratinase has the ability to degrade diverse substrates with enzymes (Harrap and Woods, 1964; Cherry *et al*., 1975).

Bio-processing of poultry (keratin rich) waste

The main component of feather is Keratin which represents 90% of feather weight and this is 10% of total weight of chicken (Harrap and Woods 1964; Cherry *et al*., 1975). From commercially poultry processing there is an increase amount of feathers which may represent a pollutant problem and need to adequate management (Shih, 1993).At present feather meal is prepared from feathers by high energy input process that is steam pressure cooking. In some last years when food is shortage feather meal which is deficient in histidine, methionine and tryptophan had been used as animal feed. (Papadopoulos *et al*., 1986; Wang and Parsons 1997). Instead of using old method steam pressure cooking to make feather meal now enzymatic hydrolysis of feather by keratinases is mostly used (Onifade *et al*. 1998; Grazziotin *et al*., 2006). When growth rate was observed between chickens, one group which fed with soyabean meal and other group which fed with feather meal and then it is fermented with Streptomyces sp and Bacillus sp plus methionine supplementation (Elmayergi and Smith, 1971). There is a major increase in digestion of total amino acid when using commercially available Crude keratinase from B licheniformis (Shih and Williams 1990; Lee *et al*., 1991; Brutt and Ichida 1999). They show in their study in which production of enzymatic hydrolysate from chicken feathers and calculating there in vitro digestibility. Feather hydrolysate was produced by using two culture methods in which one is whole culture on 60 g/l (WCH) or other culture supernatant of cultivation on 10 g/l (CSH). After determination compare Amino acid composition of hydrolysate showing in methionine, lysine and histidine. Culture supernatant hydrolysate (CSH) indicated high amount of sulphur containing amino acid. There is higher amount of Serine, leucine and glutamate in Whole culture hydrolysate while serine, arginine and

glutamate were present in high amount in culture supernatant hydrolysate. After comparison it is found that feather meal had lower amount of essential amino acids than CSH & WCH as shown in Table 2 (Grazziotin *et al*., 2006).

Table 2. 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

Leather bio-processing

Significant amount of organic waste is produced by Leather production. For per kg hide processing there is need approximately 35-40 liter of water. Recent studies exposed that during annual global processing, 9 X 10^9 liter hides and skin are processed and the liquid discharge which is released is 30-40 X 10^{10} liters. This discharge produces some problems i.e. to access water of good quality for use and during de-hairing, management of effluent and sulfide waste. (Ramasami *et al*., 1998; Ramasami *et al*., 1999; Marsal 1999).

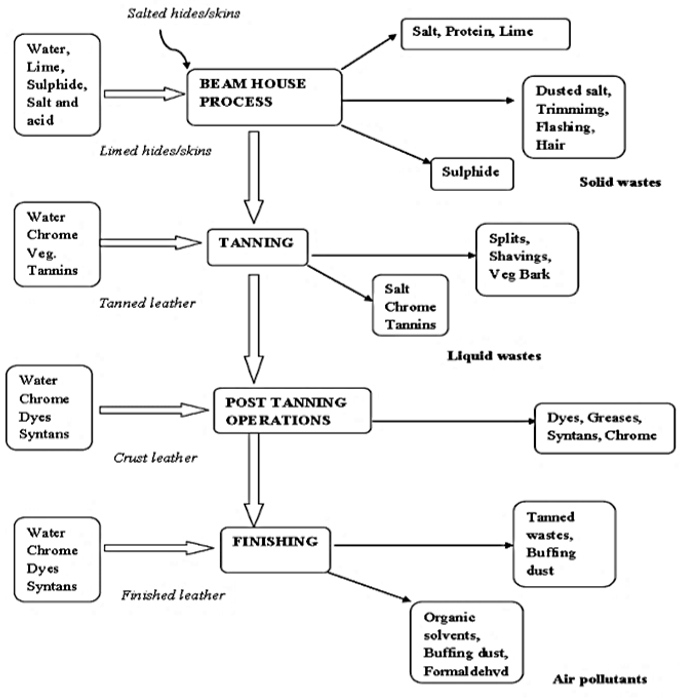

Figure 2 : Inflow and out flow of leather processing

Processing of leather involves many steps which are: 1.Pre-tanning process: cleaning of hides or skins is usually performed. 2. Tanning: In tanning stabilize skins or hides permanently. 3. Post tanning and finishing: In this step addition of aesthetic to make valuable. At each stage there is a great use of various chemicals and expulsion of wastes as shown in Fig 2. In the past Sulfide was used for de-hairing which is very toxic chemical (Rao *et al*., 1997) but now it is possible the use of sulfide instead of using enzyme assisted processes. De- hairing by Enzymes mostly uses proteolytic enzymes along with small amounts of sulfide and lime. There are different methods in which different chemicals used to de hair the hides of different animals. To de-hair bovine hides he described that mostly *Streptomyces griseus* and carbonate buffer 7 surfactant is used (Gehring, 2002). In the same way Thanikaivelan *et al*., (2004) too reported that for complete hair removal in cow skin mostly 0.5% sodium sulfide and 1% enzyme concentration used. While in Enzyme assisted de-hairing chemical usage is greatly reduced like use of sulfide concentration reduced to 85% than in conventional. And after enzymatic leather processing there is great decrease in emission and wastes are 45% and 20% respectively instead of using conventional leather processing. They reported that there is reduction in total dry sludge from 152 kg to 12 kg per 1000 kg of raw in hide processing and in this way whole enzymatic process is better than conventional process in management of total solid wastes as shown in Fig 2 (Thanikaivelan *et al*., 2002).

Bio-Hydrogen Production

There is increasing concern in utilization of renewable sources to fulfill developing energy needs of mankind (Benemann, 1996). Main purpose of production of bio-hydrogen is to make zero emission or solid wastes by producing hydrogen from biomass in photo-biological or heterotrophic fermentation routes (Cammack *et al*., 2001). Initial material for production of energy in photo-biological or heterotrophic fermentation is organic substrate. After combining two dark step fermentation Balint *et al*., (2005) isolated Bacillus strain, this strain along with anaerobic archeon (*Thermococcus litoralis*) have ability to degrade keratin and also have capability to produce hydrogen (as shown in fig 3). In their experiment they isolated a unique aerobic Bacillus strain (B licheniformis KK1) due to its high Keratinolytic

activity and this strain was engage in converting bio-waste into a highly rich amino acids and peptides fermentation product. After this in a second step of fermentation highly rich product was metabolized by *Thermococcus litoralis*, an anaerobic hyper-thermophilic archeon.They observe hydrogen production along with a clear and visible growth of *T litoralis*. Keratin hydrolysis for at least 42 h give rise to hydrogen concentration of approximately 50-70 ml H_2/h .There is a decrease production of hydrogen if hydrolysis has time less than 42 h. While considerably longer time (92 and 138 h) for proteolytic hydrolysis did not greatly disturb the capacity of H_2 production. Therefore there is recommended time for keratin hydrolysis is around 60h as it showed in Table 2 (Balint *et al*., 2005).

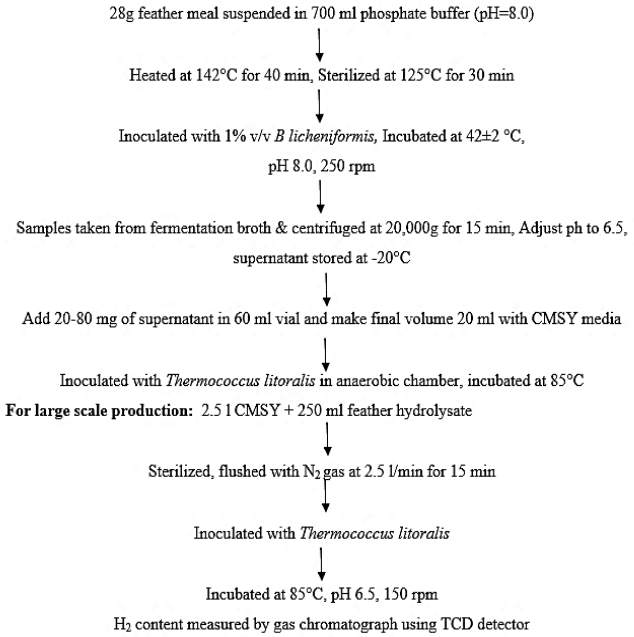

Figure 3: Flow chart of bio-hydrogen production from keratinous waste

Table 3. Overall hydrogen production

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

II. CONCLUSION

A proteolytic keratinase enzyme is used to degrade keratinous waste which mainly accumulate in environment and cause many types of environmental pollution. Keratinase is used to degrade poultry waste by producing feather meal from feathers of birds, production of hydrogen, to manage the waste of leather industry and enzymatic de-hairing in leather. Microbial keratinase has many benefits in environment for remediation of keratin than conventional method. In this review there is information about Keratinolytic microbes to manage biological wastes in environment. However there should need to increase the use of keratinase for quick removal of keratin from environment.

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