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MICROBIAL KERATINASE: A REVIEW

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ABSTRACT
Keratinases, a proteolytic enzyme act on insoluble keratin substrates and generally on a broad range of protein substrates. Keratinase attack on disulfide bond in the keratin and convert it into simple form from complex one. These enzymes have huge applications in leather industry and hydrolysis of feather and keratin. Samples collected from poultry industry wastes and soils. Keratinases are essential to form animal nutrients, protein supplements, leather manufacturing industries, textile industries, detergent formulation, feather meal processing for feed and fertilizer, the pharmaceutical and biomedical industries, and waste management. The production of microbial keratinase is less expensive than conventionally produced keratinase. Microbial keratinase can be obtained from some species of fungi, bacteria, and actinomycetes. Keratins broadly classified as hard 5% sulfur and soft 1% sulfur.

KEY WORDS: Keratinase, Producer, Production, Purification and application.

INTRODUCTION

Keratinases a proteolytic enzyme that catalyze the cleavage of keratin (Saunders *et al*., 2007). Keratin is a fibrous structural protein, in it monomers units are assemble into bundles and form intermediate filaments, which are tough and insoluble and form hard tissues which are found in reptiles, birds, amphibians, and mammals. They are affiliated as biological materials in toughness only by chitin (Hickman *et al*., 2003). There are various types of keratins within a single bioform. The α-Keratins is found in hair, wool, horns, nails, claws and hooves of animals, and the harder one β-keratins is found in nails, claws, shells, beaks, and in the feathers of birds. These keratins (β-keratins) are formed primarily in beta sheets. However, beta sheets are also found in β-keratins (Kreplak *et al.*, 2004). In keratins there is intramolecular and intermolecular hydrogen bonds, hydrophobic interactions and it also have maximum amounts of the sulfur-containing amino acid such as cystein which is required for the formation of disulfide bonds which provide extra permanent strength and rigidity by thermally stable cross linking (Lee *et al*., 2002). Now a days feathers are produced in large quantities as by-products of poultry processing industries which is main cause of environmental pollution, are the largest reservoirs of keratin. There are some traditional methods for the treatment of feather wastes, like as burning of feather, land filling, steam pressure cooking and strong alkali or acid hydrolysis which is not only cause environmental pollutions, but also require large amount of energy for processing results in destruction of some essential amino acids.

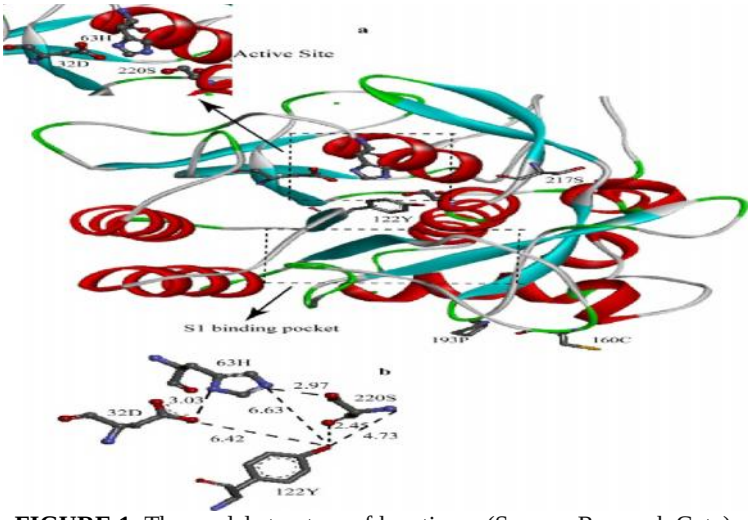

FIGURE 1: The model structure of keratinase (Source; Research Gate)

So now days, it is compulsory to development a method which should be cost effective and must be environmental friendly for the degradation of feathers. In this relation, degradation of feather by keratinase producing microorganisms signify an alternative method (Jayalakshmi *et al.,* 2012). Usually keratinases are serine or metalloproteases which degrade fibrous and insoluble keratinous materials. These enzymes are durable with broad range of temperature and pH values it has optimal activities at neutral to alkaline at temperatures ranging from 40-70^{o}C. Activities of keratinase are also broad on different types of substrates including fibrin hemoglobin, keratins, casein and gelatin. Yet, there is no equality to determine the activities of keratinase due to multiformity of the structure of keratin substrates used for such determination, and the definitions of keratinase activity (Gupta & Ramnani, 2006; Lateef *et al*., 2010). Their mode of action involves complex systems of sulfitolysis and proteolysis (Gupta & Ramnani, 2006). Keratinase is a nascent tool in various micro and biotechnological applications like as of keratin wastes converts in to animal feed, nitrogenous fertilizer, cosmetic and detergent industries. Feathers mainly composed of from above 90% protein (Onifade *et al.,* 1998) and it produces large amount of waste by poultry processing worldwide. Stockpiling of feather leads to feather protein wastage (Onifade *et al*., 1998; Gousterova *et al.,* 2005). The common species of keratinophilic fungi from soils are *Microsporum gypseum, M. canis, M. fulvum,* M. *nanum, Trichophytonterrestre, T.ajelloi, T.mentagrophytes, T.interdigitale, T.verrucosum, T. equinum, T. rubrum, T. interdigitale, T. schoenleinii, T. simii, Chrysosporium keratinophilum, C. pannicola, C. tropicum, C. indicum, C. anum, C. lobatum, C. evolceanui* and *C. indicum* (S. Shadzi *et al.,* 2002). Bacterial species which produces keratinase includes B. *subtilis* and B. *licheniformis* (V. Matikevicien *et al*., 2009), although other bacteria including Gram-positive *Lysobacter, Nesterenkonia, Kocuria, Microbacterium,* Gram negative *Vibrio, Xanthomonas, Stenotrophomonas, Chryseo bacterium, Fervidobacterium, Thermoanaero bacter,* and *Nesterenkonia* can also degrade keratin (Gupta & Ramnani, 2006). Microorganisms that produce keratinase have also important role in fermentation technology because Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into soluble protein or polypeptide (Suntornsuk and Suntornsuk, 2003).

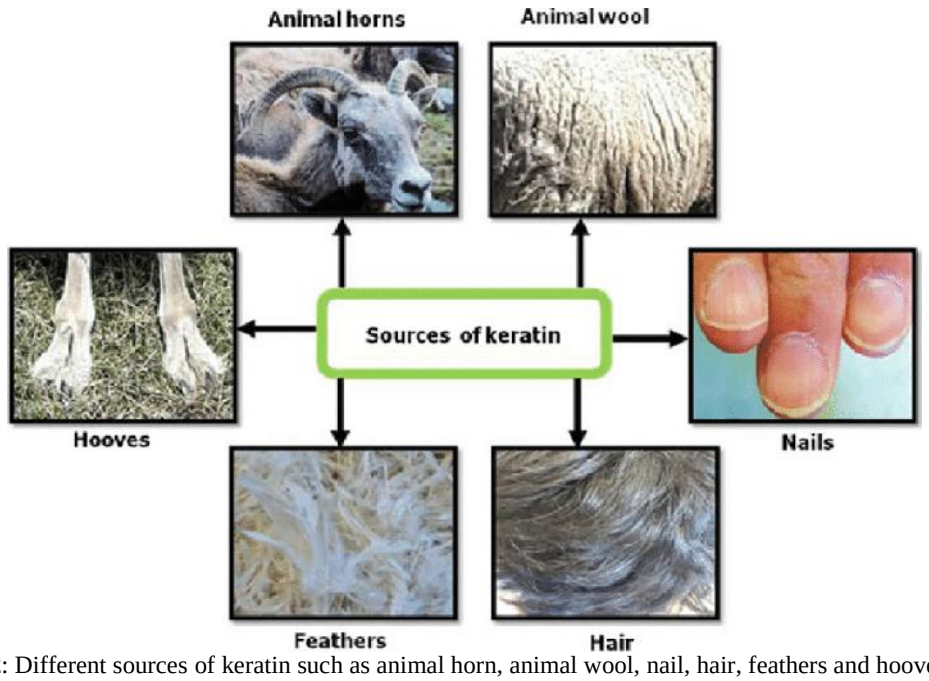

FIGURE 2: Different sources of keratin such as animal horn, animal wool, nail, hair, feathers and hooves (Source; Research Gate)

STUDIES ON KARATINASE

The Samples of soil collected from local poultry farm area. Keratinase producer bacteria was isolated from zone formation in casein agar medium and identified as *Bacillus* sp. Keratinase was confirmed using azokeratin (soluble keratin) medium (Suntornsuk and Suntornsuk, 2003). The enzyme keratinase mainly obtained from fungi, actinomycetes and bacteria (T. Korniłowicz-Kowalska and J. Bohacz, 2011). Bacterial species can grow faster than fungus and thus have efficient in industries. The advantages of fungi include easier colonization of fungal hyphae into the harder keratin relative to bacteria. The isolated bacterial strains known to degrade keratin or produce the keratinase are primarily composed of *Bacillus*; it includes *B. subtilis* and *B. licheniformis* (V. Matikevicien *et al.,* 2009).

1. To check Keratinase activity and feather degradation at different pH

Chicken feather 250 mg was transferred to 25 ml of basal medium (NH3Cl-0.5 gl-1, K2HPO4-0.3 gl-1, KH2PO4-0.4 gl-1, MgCl-0.24 gl-1, peptone-0.2 gl-1) and adjusted the pH 6.0 to 10.0 with 1N NaOH in each flask. Inoculate at 5% inoculums with the initial cell count of 5 x 107 cells ml-1 and incubated for 5 days at 37^{o}C in rotor shaker with

120 rpm. At the end of the 5th day of incubation, the culture was filtered and the dry weight of the feather was observed. The percentage of feather hydrolyzed at each pH was calculated.

2. Estimation of enzymatic activity

Keratinase activity was followed by the modified method (Yamamura *et al*., 2002). The keratinolytic activity of the mixture containing 2 ml azokeratin (1% w/v) and 0.5 ml suitably diluted enzyme was carried out at 45^{o}C for 30 min. The enzymatic reaction was stopped by adding with 2.5 ml of 10% TCA (Trichloro acetic acid) and then allowed to settle for 30 min and then filtered. To 1 ml of the filterate, 5 ml of 0.5 mM sodium bicarbonate solution and 0.5 ml of diluted Folin-Ciocalteau reagent were added. After the reaction mixture was incubated for 30 min, the absorbance was measured at 660 nm using spectrophotometer. Simultaneously a blank was read using the same steps except that 10% TCA was added prior to the addition of enzyme. Results were expressed as Keratinase units (KU ml-1) of enzyme.

3. Keratin-Degrading Bacterial Isolates

Similar to the isolates of fungi, lists of bacterial strains capable of degrading keratins have been reported. Bacteria can grow faster than fungal species and therefore have potential in industrial applications. (Sapnaand & Yamini 2011) investigated the potential degradation of keratin by bacterial strains recovered from the soil samples. Four isolates from feather waste were recovered on milk agar plates and three were identified as Gram-negative bacteria (*Burkholderia*, *Chryseobacterium*, and *Pseudomonas* species) and one was identified as Gram-positive strain (*Microbacterium* species) (A. Riffeland & A. Brandelli 2006). Moreover, T.Kornillowicz-Kowalska & J. Bohacz (2011) reported that *actinomycetes*, *Streptomyces* group, namely, *S. fradiae*, *Streptomyces* species A11, *S. pactum*, *S. albidoflavus*, *S. thermoviolaceus* SD8, and *S. graminofaciens*, as well as *Thermoactinomyces candidus*, were capable of producing keratinase.

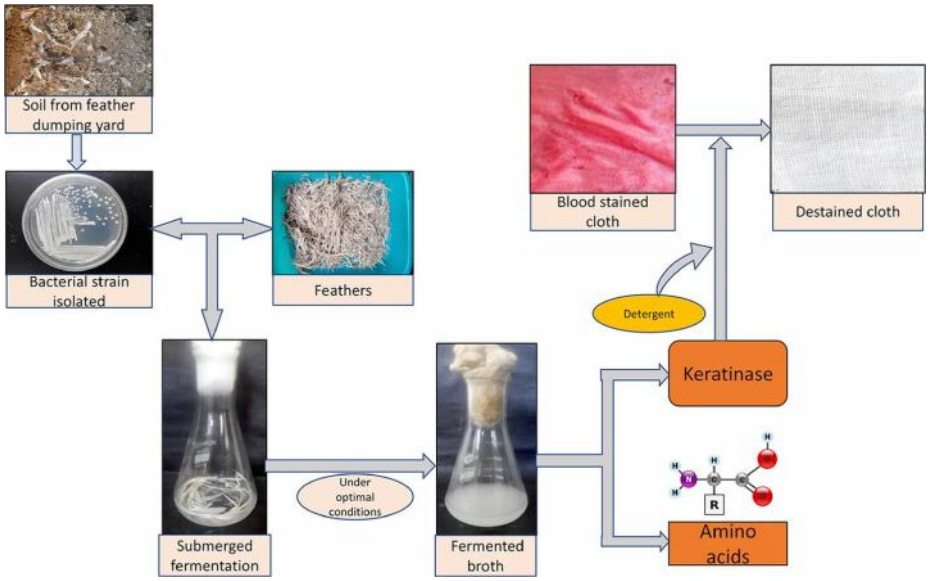

FIGURE 3: Degradation and Keratinase production (Source; Science direct.com)

4. Secretion of Microbial Keratinases

Keratinolytic enzymes are proteases known as keratinases (EC 3.4.21/24/99.11) that can primarily be obtained from fungi, *actinomycetes* and bacteria (T. Kornillowicz-Kowalska & J. Bohacz 2011). Fungal keratinases can be easily obtained by secretion, and their low cost makes them preferable over bacterial keratinases in some cases, even though the fungi grow slower and the recovery of keratinase from fungi has been reported for several decades. The availability of several strains that are capable of producing keratinase makes the situation to select efficient keratinase producers an important step. Screening microbial enzymes is essential in these Selection process, and the chosen enzymes should be less expensive, eco-friendly, and efficient. Both keratinophilic fungi and non keratinophilic fungi can produce keratinases, but the difference is the rate of production, which is higher in the former case. Several methods have been proposed to screen proteolytic (including keratinolytic activities).

5. Optimized Conditions for Microbial Keratinases

Once the microbes are isolated, then they can be further cultivated on suitable artificial growth media under optimal conditions to obtain excess production of keratinase. Sabouraud's dextrose is commonly used to grow keratinophilic fungi due to its suitability (V.M. Ramesh and A Hilda 1998; P.Anbu, A Hilda C.B.Gopinath., 2004; A.Z. Mahmoudabadi & M. Zarrin, 2008). Usually keratinophilic fungi will take a longer time to degrade the keratin (in weeks). Using the hair-baiting technique, H.C. Gugnani *et al.* (2012) found that 4 to 8 weeks were required to observe keratinophilic fungal growth.

Kumar *et al.* (2013) isolated keratinophilic fungi after 2 to 4 weeks of incubation, while Mahmoudabadi and Zarrin (2008) found that 4 to 5 weeks are necessary to grow. In such cases, optimal growth was found to occur at room temperature. It has also been reported that keratinophilic fungi are able to degrade 40% of keratin after 8 weeks,

while less than half (<20%) of that amount can be degraded in the case of non keratinophilic fungi (J. Kunest. 2000).

6. Purification of Keratinases

Purification of keratinase is necessary for further industrial applications to hasten the efficiency of keratinase action. G.S. Molyneux (1959) attempted to isolate keratinase from a bacterial source. In other cases, with the purified keratinases, several sizes were reported in the apparent molecular weight range of 27 to 200kDa from different strains of bacteria and fungi (T. Kornillowicz-Kowalska & J. Bohacz 2011). However, Kim *et al*. (2004) reported recovery of keratinase with a molecular weight of 440kDa. Purified enzymes including keratinases can be obtained using different methodologies. The most common strategy is to purify the enzymes by precipitation followed by column chromatography. Keratinase with a molecular mass of 35kDa was purified from feather degrading bacterium using ammonium sulphate precipitation followed by ion-exchange (DEAE-Sepharose) and gel filtration (Sephadex G-75). The purified keratinase was found to have thermotolerant and showed high specific activity (W. Suntornsuk, J. Tongjum, P. Onnim *et al*., 2005). Using a similar strategy, B. Zhang *et al*. (2009) purified the alkaline keratinase from *Bacillus* species and identified keratinase of 27kDa using MALDI-TOF-MS. Anbu *et al*. (2005) isolated keratinase with a molecular weight of 39kDa from the poultry farm isolate, *Scopulariopsis brevicaulis* and found that this keratinase had a serine residue near the active site. Keratinase with a size of 41 ±1kDa and activity under the optimal conditions at pH 9.0 and 50°C was isolated from *Bacillus megaterium*. This enzyme was also found to have a serine active site and to be inhibited by PMSF (V. Saibabu, F.N. Niyonzima & SS More 2013).

Based on the pH adaptation nature of the keratinase, the column matrix and method of purification can be desired while varying the elution profile.

Characterization and identification of molecular weight of the keratinase

Culture filtrate was centrifuged at 10,000 rpm for 15 min and the supernatant was precipitated by ammonium chloride or acetone. Then the precipitate was dialyzed. Around 50 µl of the precipitate was run on SDS-PAGE with standard marker.

Mode of action of keratinase:

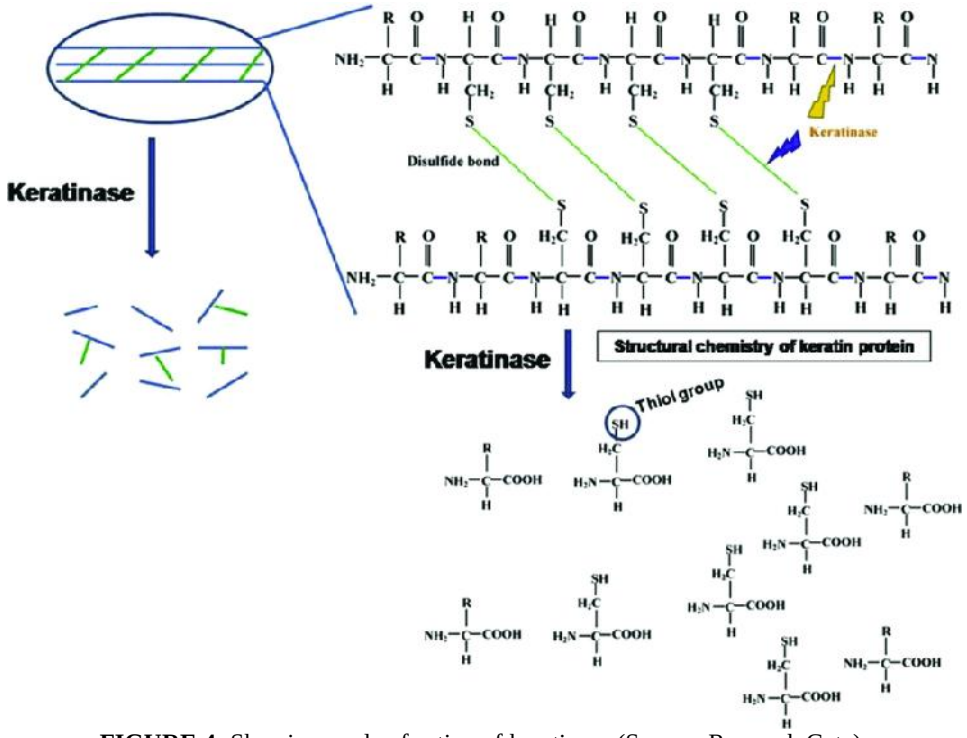

FIGURE 4: Showing mode of action of keratinase (Source; Research Gate)

Applications of keratinase

Keratinase has multidisciplinary applications in the field of cosmetic industries, leather industries, detergent industries, animal feed manufacturing industries and also in waste management. Keratinase also modify keratinous waste into useful materials such as formation of biodegradable films, glues, coatings and edible film applications (Gupta & Ramnani, 2006).

Keratin waste management

There are many microbial strains that could be useful in feather waste management which is main byproduct of poultry wastes as keratinase have great feather degradating ability (Lateef *et al.,* 2010). In meat industries elastin, Collagen, keratin and proteins originate as wastes in meat industry efficiently degraded by the keratinolytic enzyme E77 (Zhao *et al*., 2012). *Stenotrophomonas maltophilia* strain that produce keratinase is used to decompose wool waste (Fang *et al.,* 2013). So, there is huge application of

keratinases for the treatment of slaughterhouses and waste management.

Cosmetic and pharmaceutical application

Keratinases are obligate biocatalysts in cosmetic and pharmaceutical industries. They have been mentioned as an ingredient in depilatory formation for hair shaving creams and skin lightening agents (Yang, 2012). Crude keratinases also used to raise hair qualities like as, volume of hair, brightness, flexibility, softness and strength of hair so; it can be employed as hair care products (Cao *et al.*, 2012). Beside this, keratinases believable to degrade thick dead layered skin of toes and fingers (hyperkeratosis) thus serve as a workable substitute to the traditional method of using salicylic acid (Gupta & Ramnani, 2006). In spite of this, keratinases are able to peel of skin to remove acnes, which are due to blockage of sebaceous gland by keratins (Selvam & Vishnupriya, 2012). This enzyme also degrade Prions PrPSc, an infective protein molecules which is responsible to cause a disease called scrapie a disease of sheep, mad cow disease or bovine spongiform encephalopathy kuru, a rare disease caused by an infectious protein found in contaminated human brain and Creutzfeldt-Jacob disease (CJD) (Caughey, 2001). Although, keratinase have been used to increase drug delivery through topical application, to minimize systemic side effects. The existence of keratinase has been remarked to increase drug ingression via the nail plate, which is a barrier to ingression of some other drugs (Mohorcic *et al*., 2007). Keratinase also be used to disinfect laboratory apparatus and medical equipment due to their prion protein degrading ability (Liang *et al*., 2010).

Production of animal feed: Dietary keratinase is used to lift up immune response, improve nutrient digestibility, helps to gain weight, reform intestinal morphology and ecology in growing and nursery pigs (Wang *et al.*, 2011a). The hydrothermal treatment of keratin substrates reduces its nutritional value, as it destruct certain types of essential amino acids such as, histidine, lysine, methionine, and tryptophan, and also hydrothermal treatments are enables to release certain amino acids from the keratins. Thus, the use of keratinases/ keratinolytic microorganisms is a good alternative. A keratinolytic strain of B. *subtilis* was also reported to produce proteinous hydrolysate of high antioxidative potential from wool waste (Fakhfakh *et al.*, 2013). To cast aside the potential of infection from microorganisms that may be pathogenic the use of enzyme keratinase for degradation of feather is more beneficial than microbial degradation.

Leather processing industries

It is the best method to remove hair from skin without harming the skin and hair fragment so dehairing by keratinase proven best alternative method. This method is cheap, easy and protective and also does not cause any harmful impact on environment. There are many statements of dehairing of animal skins by microbial keratinases (Rai & Mukherjee, 2011; Paul *et al*., 2013a; Chaturvedi *et al*., 2014).The combination of lipases, proteases and carbohydrases have been used as a biocatalysts for dehairing process. Keratinolytic proteases without help of collagenolytic and elastinolytic activities are good for dehairing process (Gupta & Ramnani, 2006). The keratinase of a strain of Bacillus safensis LAU 13 completely dehaired goat skin within 12 h without noticeable damage to the skin, whereas incomplete dehairing with skin damage was obtained using chemical-based method (Lateef *et al*., 2015a). Now a days chromium sulfate is widely used worldwide (Subrama naidu A 1995) as a tanning agent due to its versatile nature to produce different types of leathers with required properties and uses (chandrasekasekaran *et al*.,1999) (Chattapadhyay B *et al*.,2000). But this process is pollution causing and toxic in nature. However, the keratinase application are ecofriendly for processing of leather and skin products.

Production of nitrogen fertilizer and biofertilizer

Poultry waste Feathers are recycled by keratinase to produce slow-release nitrogen fertilizer. (Paul *et al*., 2014a) which are better to increase soil fertility without harming it.

Textile industry

Keratinase also have great importance in textile industry because keratinases are able to improve silk and wool quality in textile industries. (Cai *et al*., 2011) treated wool and polyester-blended fabrics with crude keratinase which was isolated from pseudomonas strain, and it was experienced that the fabrics become shrink resistance and also its tensile strength increases.

In detergent industries

The keratinolytic proteases will put more to the value of proteolytic enzymes in detergent formulation due to their ability to degrade insoluble keratin and their properties like stability at high temperature and pH, activity at wide range of temperature and pH, stability in the presence of surfactants, oxidizing and bleaching agents, chelating agents and compatibility with some commercial laundry detergent (Gupta & Ramnani, 2006). In a study, keratinase of *Paenibacillus woosongensis* TKB2 was combined with detergent and safely removed blood, fruit juice and turmeric stains from fabric (Paul *et al*., 2013b). Also, (Paul *et al*., 2014b) reported the safe removal of blood, egg yolk and chocolate stains from cloths by crude keratinase. The keratinase was stable in the presence of EDTA, and the preparation of enzyme beads using 1.5 % CMC significantly improved its storage stability. Thus, keratinases are important enzymes that can be used as additives in detergent formulations for efficient removal of keratinous wastes in an eco-friendly manner.

CONCLUSION

At present the applications of keratinase is very vast. It is used for many applications such as bioremediations, waste managements, cosmetic industries, pharmaceutical industries, textile industry, leather industries etc. Thus, it is also used in agricultural purpose to make biofertilizer, composed and biopesticides. Thus, keratinase play a multidisciplinary role for human welfare. To drive these uncountable applications of keratinase, search for new sources of keratinases would be sustained, New techniques should be develop to increase enzyme production and its cost production should be low. The multi-functionality of keratinases will strengthen research efforts that would lead to the production and creation of novel products of biotransformation, which will continue to define bioresource utilization of keratinous wastes.

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