CHAPTER – II

LITERATURE REVIEW

2.1 SOURCES OF KERATIN

Keratin is one of the most abundant biopolymers in the world (Martinez-Hernandez, et al., 2012). It is a tough, fibrous, insoluble material that functions as an outer coat of human and animal organs, to prevent the loss of body fluids. Keratin is predominantly found in tissues of reptiles, birds, amphibians and mammals. The structural component of feathers, hair, nails, horns, hooves, bones, furs, claws, hides, bird beaks, skin, wool and scales is made up of keratin. α-keratins (alpha-helix) are usually found 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 expressed in the epithelial cell types of digestive organs (liver, pancreas, intestine and gall bladder), which include hepatocytes, hepatobiliary ductal cells, oval cells, acinar cells, enterocytes of the small intestine, colon and goblet cells (Fortier, et al., 2012).

Keratin is rich in sulphur compounds with disulphide bridges, which imparts them the insoluble nature. It also contains a variety of amino acids, predominantly cystine, lysine, proline and serine. Keratin is hard containing scleroprotein which is unreactive against most chemicals and is not digested by pepsin, trypsin or papain. (Kanchana, 2012). Keratin utilization has been reported in variety of organisms including non-filamentous and filamentous bacteria, water moulds and filamentous fungi. Along with bacteria and fungi, some insects including cloth mouth leaves, carpet beetles are known to digests keratin. (Bin Zhang, et al., 2009).

2.2 KERATIN STRUCTURE

Keratins are fibrous proteins which compose the structures and large portions of the cell compositions of living organisms (Lutz Langbein et al., 2001). Fig 2.2.1 shows the molecular structure of keratin. There are two primary groups of keratins, the alpha-keratins and the beta-keratins while both fulfill similar roles, they differ slightly in structure, composition and properties. The alpha-keratins
are slightly basic or neutral and a right handed helical structure and the beta keratins are slightly acidic and also forms a right handed helical structure. Keratins are composed of amino acids, primarily the amino acid glycine and alanine (Hideto Takami, et al., 1999).

Fig. 2.2.1 Molecular Structure of Keratin (Bradbury, 1976).

2.3 MICROBIAL HYDROLYSIS OF KERATIN

Bernal. et al., 2003 identified the strain of kocuria rosea and was able to secrete keratin hydrolyzing Proteinase (keratinase) in submerged batch cultures with finely milled feathers as carbon & nitrogen source was studied. The maximum production of Keratinases was 17 U/mg when feathers were used as the only fermentation substrate (17u/mg), the optimum temperature & pH was 40 °C & 10.0 respectively, considerably lower activity was present in cultures containing glucose & others nutrient supplements. Biodegradation of feathers by this microorganism could be a useful biotechnological tool in the leather, food and cosmetic industries. Cultivation conditions affecting feather degradation by Bacillus sp. fk 46 were investigated by Suntorsuk, et al, 2003 and the results showed that feather was almost completely degraded under the following conditions; 1(%w/v) whole chicken feather as a substrate at the initial medium pH of 9.0 with 5% bacterial inoculums, at a temperature of 37 °C & shaking
speed of 250 rev/min. After feather was degraded, its residue and fermented broth became a protein feed for animals.

Allpress, et al, 2002 have pointed out nine screened bacteria were reported to produce extra cellular protease, for production of keratinases. Of these, *Lysobacter NCIMB 9497* exhibited the maximum keratinolytic activity. Optimum activity occurred at 50°C which indicates that keratinase is a metallo protease with a high degree of keratinolytic activity and stability. Degree of keratinolytic activity and stability indicate considerable biotechnological potential in the leather industry, and in the processing of poultry waste.

An analysis by Letourneau, et al., 1998 shows that *Streptomyces sp*. Producing a high keratinolytic activity when cultured on feather meal medium was isolated from a naturally degraded feather. Maximal keratin degradation was observed at 70°C and pH 10.0. Comparison between proteolytic activity derived from this new strain (S.K 1-02) and commercial proteases indicated that SK1-02 could be a useful biotechnological tool in the valorization of keratin containing wastes, or the depilation process in the leather industry.

Suntorsuk, et al., 2003 utilised the *Bacillus sp FK 46* inoculated medium containing different concentration of feather (1-5%). Feather concentration affected bacterial growth & feather degradation. Bacterial growth increased as the feather concentration increased, while the percentage of feather degradation was inversely proportional to the feather concentration. Keratinase was, however, produced at a similar level at different feather concentrations. It was demonstrated that high feather concentration may cause substrate inhibition or repression of keratinase production, resulting in a low percentage of feather degradation.

Similar result was given by Mohamedin, 1999 utilising *S. thermonitrificans* grown on a basal liquid medium containing different concentration of feather pieces and incubated for 96 hours and 50 ºC. Proteolytic activity increased as the amount of feather increased (0.25-1.5%); but when the concentration was raised to 2.0% enzyme production decreased. It reached their maximum values at 1.5% ( w/v) of feather concentration and decreased thereafter. An isolated *Vibrio Sp*. Strain Kr producing a
high keratinolytic activity on feather-containing broth was identified by Sangali et al., 2000; and was active on azokeratin, azocaesin and arginine-p nitro anilide.

Report given by Riffel, et al., 2002 shows *Flavobacterium* species producing high keratinolytic activity was isolated. This grew on feather meal broth, producing keratinase and was also capable of complete degradation of raw feathers. Research studies by Bockle, et al., 1995 reveal a keratinolytic serine protease from *Streptomyces pactum* DSM 40530 was characterised. The enzyme was purified by casein agarose affinity chromatography and had a molecular weight of 30,000 Daltons. The proteinase was optimally active in the pH range from 7.0 to 10.0 and at temperatures from 40 to 75°C. Earlier researcher Friedrich et al., 1999 found that almost some 300 common fungi were screened for the synthesis of extra cellular keratinases, About 54% of the fungi grew on agar plates with soluble keratin and excreted the enzymes and found that *Aspergillus flavus* was the most powerful producer of extra cellular keratinase when cultivated in submerged condition.

### 2.4 BIOPROCESSING KERATIN-RICH WASTE

The use of keratinolytic microorganisms arose as an important alternative for recycling keratinous by products, particularly from the poultry and leather industries. The developments of bioprocesses that can convert the huge amounts of such by products into added-value products have been investigated (Grazziotin, et al., 2007). The ability of most keratinases to hydrolyze substrates (Lin, et al., 1992) indicates the potential of such enzymes for bioconversion of keratin wastes to feed use or other value added products. Keratin represents nearly 90% of feather weight, which constitute up to 10% of the total chicken weight. 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 to feather meal by steam pressure cooking, which require high-energy input. Feather meal has been used on a limited basis as an ingredient in animal feed, as it is deficient in methionine, histidine, and tryptophan (Papadopoulos, et al.,1986). The use of Keratinase to up grade the nutritional value of feathers or feather meal has been described (Onifade, et al., 1998).
The use of keratinolytic bacteria for the production of feather hydrolysates has been the subject of some patented processes (Burt and Ichida, 1999 and the keratinase from \textit{B. licheniformis PWD-1} is commercially produced under the trade name versazyme. Production of keratinases using feather meal or raw feathers as the basis for culture media has been described and several factors such as pH, feather concentration, inoculum and temperature can influence the resulting enzyme yield (Wang and Shih, 1999). More recently, the use of statistical optimization by response surface methodology has been described for the production of bacterial keratinases during growth on raw feathers (Ramnani and Gupta, 2004). Leather production yields significant quantities of organic waste a significant portion of which comes from degraded keratin. Biotechnological options are available for handling effluents and proteinaceous solid waste (Thanikaivelan, et al., 2004). Biodegradation of waste from the leather industry has been reported, as the ability of \textit{Streptomyces} and \textit{Bacillus} to hydrolyze hair and wool keratins has been already described (Takami, et al., 1992b). The bacterium \textit{S. fradiae} substantially degrade the complex morphological structure of wool, hair and feather substrates by a combination of mechanical and enzymatic activity resulting in the release of soluble protein during fermentation (Hood and Healy, 1994). Sharma and Berwick, 1991 described the construction of a rotating frame bioreactor in which wool substrate was almost totally solubilized by \textit{S. fradiae}. The Feather-degrading bacteria to accelerate the composting of dead chickens or feather waste could be an economical and environmentally safe method of recycling these organic materials into high-nitrogen fertilizers (Ichida, et al., 2001).

\textbf{2.5 EARLY USES OF KERATINS}

The earliest documented use of keratins for medicinal applications comes from a Chinese herbalist named Li Shi-Zhen in the 16th century. Substance made of ground ash from pyrolized human hair was used to accelerate wound healing and blood clotting called Xue Yu Tan, also known as Crinis Carbonisatus. Although the details about the discovery of the biological activity of human hair are not reported in great detail, its uses for medicinal purposes are clearly documented. The word “keratin” first appears in the literature around 1850 to describe the material that made up hard tissues such as animal horns and hooves (keratin comes from the Greek “kera” meaning horn). At the time, keratins intrigued scientists because they did not behave like other proteins.
In particular normal methods for dissolving proteins were ineffective for solubilizing keratin. Although methods such as burning and grinding had been known for some time, many scientists and inventors were more interested in dissolving hair and horns in order to make better products. John Hoff Meier described a process for extracting keratins from animal horns using lime. The extracted keratins were used to make keratin-based gels that could be strengthened by adding formaldehyde. During the years from 1905 to 1935 many methods were developed to extract keratins using oxidative and reductive chemistries. These technologies were initially applied to animal horns and hooves, but were also eventually used to extract keratins from wool and human hair. The biological properties of the extracts led to increased interest in the development of keratins for medical applications and among the first inventions were keratin powders for cosmetics, composites and coatings for drugs.

During the 1920s, keratin research changed its focus from products made from keratin to the structure and function of keratin proteins. Scientists soon concluded that many different forms of keratin were present in these extracts and that the hair fiber must be a complex structure, not simply a strand of protein.

2.6 KERATIN RESEARCH FROM 1970-PRESENT

Advances in the extraction, purification and characterization of keratins, led to the exponential growth of keratin materials and their derivatives. In the 1970s, methods to form extracted keratins into powders, films, gels, coatings, fibers, and foams were developed. All of these methods made use of the oxidative and reductive chemistries developed decades earlier, or variations thereof. The prospect of using keratin as a biomaterial in medical applications was obvious. During the 1980s, collagen became a commonly used biomolecule in many medical applications. Other naturally derived molecules soon followed such as alginates from seaweed, chitosan from shrimp shells, and hyaluronic acid from animal tissues. The potential uses of keratins in similar applications began to be explored by a number of scientists. In 1982, Japanese scientist described the use of a keratin coating on vascular grafts as a way to eliminate blood clotting, as well as experiments on the biocompatibility of keratins (Jillian Rouse, et al., 2010).
2.7 SOURCES OF KERATINOLYTIC ENZYMES

Onifade, et al., 1998 observed that most keratinases are largely inducible requiring keratin as an exogenous inducer. A number of microorganisms have been reported could degrade different sources of keratin, mainly bacteria, actinomycetes, saprophytic and dermatologist fungi have been reported to exhibit keratinolytic properties. Keratinases from many bacteria have been isolated and characterized. For instance, keratinase from *Bacillus sp.* such as *B. Licheniformis* (Ramnani, et al., 2005; *Pseudomonas sp.* (Brandelli and Riffel, 2006), *Microbacterium* (Thys, et al., 2004), *Streptomyces sp.* (Bockle and Muller, 1997; Bressolier et al., 1999; Letourneau et al., 1998)) and *Stenotrophomonas sp.* (Yamamura et al., 2002). These enzymes are produced by keratinolytic bacteria that are mostly reported by species of *Streptomyces* and *Bacillus*. The keratinases are mainly serine proteases with molecular weight ranging from 20 to 50 K Da and most of them are optimally active at temperatures up to 50°C, although a number of thermophilic keratinases are described.

Similarly Siesenop, et al., 1995 provide a detailed analysis of the duration and intensity of keratinase secretion were strongly influenced by various keratinous substrates. Wei-Hsun Lo, et al., 2012, provide an insight to the study of different keratinous substrates such as commercial feather meal, chicken feather, human hair and goose feather were used to investigate the effect of the keratinous substrate on the keratinase production by *B. cereus WU2* and the highest keratinase activity (1.75 KU/ml) was observed with chicken feather powder and whole chicken feather was the second (0.75 KU/mL). Singh et al., 1997 observed keratinase induction by various keratinous substrates with *Trichophyton simii* which was induced by buffalo skin and human nails to produce keratinase. Experimentation by Muhsin, et al., 2001; Besides, *Trichophyton mentagrophytes var. erinacei* showed the highest keratinase production with wool and *Aspergillus flavus* with chicken feather and keratinase activity was highest for *C. pannicola* and *M. Gypseum* in a culture medium induced with human hair.
2.8 KERATINASES

Keratin is an insoluble and fibrous structural protein that is a constituent of feathers and wool. The protein is abundantly available as a by-product from keratinous wastes, representing a valuable source of proteins and amino acids that could be useful for animal feeds or as a source of nitrogen for plants (Gushterova, et al., 2005). However, the keratin-containing substrates and materials have high mechanical stability and hence are difficult to be degraded by common proteases. Keratinases are specific proteolytic enzymes which are capable of degrading insoluble keratins. The importance of these enzymes is being increasingly recognized in fields as diverse as animal feed production, textile processing, detergent formulation, leather manufacture and medicine. Proteolytic enzymes with specialized keratinase activity are required to degrade keratins, and for this purpose the keratinases have been isolated and purified from certain bacteria, actinomycetes, and fungi. Keratinases have been classified as serine or metallo-proteases. Cloning and expression of keratinase genes in a variety of expression systems have been reported.

Baihong, et al., (2013) described the enhanced thermo stability of a preparation of keratinase by computational design and empirical mutation. The quadruple mutant of Bacillus subtilis had been characterised to exhibit the synergistic and additive effects at 60 °C with an increase of 8.6-fold in the t1/2 value. The N122Y substitution also led to an approximately 5.6-fold increase in catalytic efficiency compared to that of the wild-type keratinase. Indhuja, et al., 2012 reported that an alkalophilic strain of Streptomyces albido flavus had produced extracellular proteases. This particular type of protease was capable of hydrolysing keratin. The biosynthesis of this specific enzyme was optimized in submerged batch cultures at highly alkaline pH 10.5 and the enzyme yield was stimulated by using an inducer substrate containing keratin in the form of white chicken feathers.

2.9 STRUCTURE OF KERATINASES

The chemical structure of Fervidobacterium pennivorans keratinase, Fervidolysin, mapped by crystallographic analysis was shown in Fig 2.9.1 with 1.7 Å resolution suggests that it is composed of four domains; a catalytic domain (CD), propeptide domain (PD) and two β- sandwiched domains (SD1 and SD2) held together by hydrogen bonds and hydrophobic interactions (Kim et al, 2004). It was
further suggested that keratinases of molecular weight less than 35 KDa may contain only the catalytic domain (Kim, et al., 2004). The Structure of the complex of *streptomyces griseus* keratinase and the third domain of the turkey ovomucoid inhibitor at 1.8- Å resolution was shown in Fig. 2.9.2 (Read, et al., 1983)

### 2.10 BIOCHEMICAL PROPERTIES OF MICROBIAL KERATINASES

Table 2.10.1 gives the biochemical characteristics of selected keratinases. Lin et al., 1992 observed the keratinase cannot only hydrolyse keratin but has the ability to hydrolyse several protein but has the ability to hydrolyse several protein substrates, for keratinases of *Bacillus licheniformis*; and Friedrich and Kern, 2003 with *Doratomyces microsporum*. Riffel, et al., 2003b investigated the low hydrolysis of collagen and elastin is a desirable property for depilatory purposes. Indeed, it has been demonstrated that a crude protease of *Chryseobacterium sp.* was able to de-hairing bovine pellets. Analysis by Cohen, et al., 2003 shows collagen and elastin are similar to keratin in that they are insoluble, highly structured and resistant to many proteases. However, examination of the individual structures of these proteins reveals quite different assemblies. Collagen like keratin, is a fibrous protein and consists of three polypeptides, called α-chains, which wrap around each other in a triple helix conformation, forming a rod-like shape. Jones, et al., 1997 studied the resistance of keratin was due to the strong intermolecular disulphide bonds and other molecular interactions, and also to the super coiled helical structure of the protein chains. These properties provide some degree of resistance to peptide hydrolysis.

Dastager Syed, et al., 2009, reported the enzyme had an optimum activity about 1.4 U/ml) at 45°C and was rapidly inactivated at higher temperatures. Enzyme was stable at 60°C and was no longer active between 60°C. It was observed that a free form of purified enzyme was less stable than an enzyme-substrate mixture under high temperature incubation. The keratinase was active in neutral and alkaline conditions with an optimum activity at pH 9.0 (1.39U/ml), depending on the buffer used. It was stable over a wide range of pH values, with the highest stability at pH 7-9, and the activity was improved in basic conditions. The keratinase was significantly inhibited by cacl₂ and partly inhibited by EDTA whereas, Na₂SO₃ enhance the enzyme activity by 2.9 times more. The enzyme degraded keratins from different sources. However, it hydrolysed keratins less than soluble proteins such as casein, bovine serum albumin and gelatin.
Fig. 2.9.1 Crystal Structure of Keratinase (Kim et al, 2004).

Fig. 2.9.2 Crystal Structure of the *streptomyces griseus* Keratinase (Read, et al, 1983)
<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Catalytic type</th>
<th>Mol.mass (KDa)</th>
<th>Optimal pH</th>
<th>Optimal Temperature (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. halodurans</em> PPKS-2 Keratinase-1</td>
<td>Disulphide reductase</td>
<td>30</td>
<td>11.0</td>
<td>60-70</td>
<td>Patange Prakash et al., 2010</td>
</tr>
<tr>
<td><em>Bacillus sp</em> Keratinase – 2</td>
<td>Serine protease</td>
<td>66</td>
<td>11.0</td>
<td>70</td>
<td>Prakash et al., 2010</td>
</tr>
<tr>
<td><em>Bacillus sp</em> JB 99</td>
<td>Serine protease</td>
<td>66</td>
<td>10.0</td>
<td>65</td>
<td>Pushpalatha 2010</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em>, MS21</td>
<td>Serine</td>
<td>30</td>
<td>8.0</td>
<td>37</td>
<td>Turks et al., 2010</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> Vibrio sp.Kr 2</td>
<td>Serine</td>
<td>33</td>
<td>7.0</td>
<td>50</td>
<td>Richa Sharma et al., 2010</td>
</tr>
<tr>
<td><em>Doratomyces microspores</em> Streptomycyes sclerotialus</td>
<td>Serine</td>
<td>45-70</td>
<td>9</td>
<td>-</td>
<td>De Toni et al., 2002</td>
</tr>
<tr>
<td><em>Streptomyces strain</em> BA 7 Aspergillus oryzae NRRL-447</td>
<td>Serine</td>
<td>44</td>
<td>8.5</td>
<td>50</td>
<td>Richar. J et al., 2005</td>
</tr>
<tr>
<td>Ahm 1</td>
<td>Metallo</td>
<td>19</td>
<td>6-8</td>
<td>50</td>
<td>Yadav A.K. et al., 2011</td>
</tr>
<tr>
<td>Ahm 2</td>
<td>Protease</td>
<td>40</td>
<td>6-11</td>
<td>60-65</td>
<td>Patange Prakash et al., 2010</td>
</tr>
<tr>
<td><em>Chrysobacterium sp.strain</em> Kr 6</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>50-60</td>
<td>Silvana Tera Silveira, et al., 2010</td>
</tr>
</tbody>
</table>
2.10.1 Physicochemical Properties of Human Hair

Hair is surprisingly strong. Cortex keratin is responsible for this property and its long chains are compressed to form a regular structure. The physical properties of hair involve stretching, elasticity and hydrophilic power. Generally physical properties of hair depend on its geometry (Valeria et al., 2009). Due to elasticity, hair can resist forces that could change its shape, its volume or its length. Elasticity is one of the most important properties of hair. Hair fibre has an elastic characteristic and it may undergo moderate stretching. The elasticity of hair depends on the long keratin fibres in the cortex. Both natural sunlight and artificial ultraviolet light break down chemicals in the hair and damage its elasticity. The texture of hair depends mainly on average diameter of the individual hairs. There are different chemical components present in the human hair it is an “integrated” system and act together to maintain the normal flow of functions.

The chemical composition of hair fibre includes essential functional elements like amino acids, keratin, melanin, and protein. Proteins are present also within the cuticle which provides elasticity to the hair. Hair from its growth under the skin of the scalp, is filled with a fibrous protein called keratin. The keratin protein found in hair is called hard keratin. It is made up of eighteen amino acids. The lipid content of the hair is not constant but varies with age and other factors. In the hair structure, lipids are present in Inner Root Sheaths and hair shaft lipids provide sheen to the hair and contribute towards its tensile properties. Melanin is the hair pigment which gives color to the skin and hair. The size, type and distribution of the melanosomes will determine the natural color of the hair (Juez & Gimier, 1983).

Amino acids are the principle building block of the keratin proteins found in hair fibres and approximately 20 different amino acids are present in these proteins. The chemical composition of hair fibres is dominated by carbon, which comprises about 45% of the atomic structure of hair. Oxygen accounts for approximately 28%, nitrogen 15%, hydrogen 7%, and sulfur 5% and several essential trace elements are also present in hair fibres including iron (20 - 220 ppm), copper (10-20 ppm), zinc (190 ppm) and iodine (0.6 ppm) (Wella, 1999).
2.10.2 Mechanical Properties of Hair

The mechanical properties of $\alpha$-keratin fibres such as hair fibres and wools are primarily related to the two components of the elongated cortical cells, the highly ordered intermediate filaments (micro fibrils) which contain the $\alpha$-helices, and the matrix in which the intermediate filaments are embedded (Feughelman, 2002). Mechanical properties such as elasticity and durability are governed by the interactions of proteins in the cortex. The cortex is a complicated, disulfide cross-linked polymer system comprising the crystalline low-sulfur proteins and the globular matrix of high-sulfur proteins.

2.10.3 Physicochemical Properties of Feather

The moisture content of processed feathers can vary depending upon processing and environmental conditions. The moisture content of feather fibre is an important variable that can have implications ranging from transportation costs to mechanical properties. Hong and Wool, (2005) reported a typical value of 8 mm for fibre length. Density of solid keratin was studied by Arai, et al., (1989) and also Barone and Schmidt, (2005) reported an apparent density of feathers and reported fibre lengths of 3.2-13 mm for the feather fibre. Hong and Wool (2005) have reported that the density of feather fibre is 0.8 g/cm$^3$. The structure of keratin, affects its chemical durability which is the primary constituent of feathers. Keratin shows good durability and resistance to degradation.

2.10.4 Mechanical Properties of Feather

The mechanical properties of bird’s feathers are highly related to structure of keratin and their functions (Bonser & Purslow, 1995). Further, work carried out by Cameron, et al., (2003) confirmed that the mechanical properties of feather keratin vary appreciably along the length of the rachis. Using x-ray diffraction, Cameron et al., 2003 discovered that, moving from calamus to tip, the keratin molecules become more aligned than at the birds skin before returning to a state of higher disorder towards the rachis tip. Purslow and Vincent, 1978 measured the elastic modulus of feather rachis from pigeons with and without inner quill. Taylor, et al., 2004 studied the effect of moisture content on mechanical properties.
2.10.5 The pH and thermal stability of keratinases

Aida Farag, et al., 2004, studied the optimum pH of the free keratinase enzyme and immobilised enzyme, the results indicated that high pH 10.7 does not decrease Keratinase activity of both forms but acidic pH does, this is in good agreement with previous results (Lin, et al., 1996). The effect of pH on activity of the keratinase enzyme was investigated by Anbu, et al., 2005, which shows the keratinase was active in alkaline condition, with optimal activity at pH 8.0 and the activity was found to decrease as pH was increased above the optimum. Similar results were reported previously (Gradisar, et al., 2000, Brouta, et al., 2001 & Mignon, et al., 1998). The result obtained by the authors Refai, et al., 2005 were in harmony with previous reports (Lin, et al., 1992, Nitisinprasets, et al., 1999 & Gessesse, et al., 2003).

The maximum keratinase activity of *B. Pumilus* was achieved at pH 9. Lonata, et al., 2008 reported that the keratinase from *Clostridium* strain PE, as other similar enzymes from bacteria, actinomycete and fungi, has a pH optimum in a neutral to alkaline range (Bockle, et al., 1995; Bressollier, et al., 1999; Friedrich, et al., 1996). However, the results are similar to *Chryseobacterium* sp.Kr6 Protease (pH 8.5), Riffel, et al., 2007 studies shows that the above enzymes showed maximum activity in alkaline medium and are similar to most of the keratinases (Nam, et al., 2002; Bernal, et al., 2006; Nilegaonkar, et al., 2007). The stimulus of keratinase activity by alkaline pHs suggests a positive biotechnological potential. Alkaline proteases have been extensively used to formulate detergents, leather industry and in bioremediation process.

The results of the study by Periasamy Anbu, et al., 2008, supported the optimum pH of 7.8 was similar to the keratinase of *M. canis* (Brouta, et al., 2001), *D. microsporus* and *P. marquandii* (Gradisar, et al., 2000). Contrary to this, the pH optimum of the Keratinase from *T. schoenleinii* and *T. mentagrophytes Vareinacei* was 5.5 (Qin, et al., 1992; Muhsin and Aubaid, 2000). Radhika Tatineni, et al., 2008 studied the production of Keratinase using *Streptomyces S7* species and reported the pH optima as 11. Geun-Tae Park and Hong-Joo son, 2009 have indicated that *B. Megaterium F7-1* was a novel alkali tolerant bacterium, which could be more efficiently applied to the alkaline condition.
Similar results from previous studies showed that the *B. licheniformis* PWD-1 exhibited maximum keratinase activity under neutral conditions (Wang and Shih, 1999), whereas *Bacillus pumilus* gave keratinase activity in a weekly acidic environment (Kim, et al., 2001).

Keratinase production of *Vibrio* Strain Kr2 and *Chryseobacterium* strain Kr6 was observed at pH 5.0-8.0 (Sangali and Brandelli, 2000; Riffel, et al., 2003). Anapaula, et al., 2010, showed the maximum activity of the keratinase from *Bacillus Sp.*P7 was observed at pH 9.0-10.0, and relative activities above 90% were maintained at pH 8.0-12.0, indicating its predominant alkaline character. Additionally, the relative enzyme activity was higher than 75% even at acidic and neutral conditions, indicating the potential versatility of such enzyme preparations for diverse applications. Maximum activity in the alkaline range suggests a positive biotechnological potential in the detergent and leather industries (Gupta, et al., 2002).

Harde et al., 2011 reported the maximum enzyme production was obtained at a pH of 7.0 to 9.0 and was observed that maximum keratinase production occurs at alkaline pH. It may be brought out that previous studies indicated during production of keratinase, keratin utilization occurs more rapidly and to a great extent at pH 7.5 (Suntornsuk and Suntornsuk, 2003). Friedrich and Antranikian (1996) described maximum keratinase production at alkaline pH. Alkaline pH favours keratin degradation at higher pH, probably by modifying the cysteine residues to lanthionine and making it accessible for keratinase action. Ponnuswamy Vijayaraghavan, et al., 2012 studied the keratinase enzyme production using *Actinobacterium* sp. demonstrated optimum enzyme activity was at alkaline pH. Similar finding was reported by De Azeredo, et al., 2006 in case of *S.species*. Sunil More, et al., 2013, studied the production of keratinase using *C. chinulata* and found that the optimum pH as 4.5 and 10.0 signifying that the enzyme could be used in both acidic and basic pH. This could be because *C. echinulata* produces two isoforms of keratinase. Similar results were obtained by Korkmaz, et al., 2004; which indicated that the *Bacillus licheniformis* enzyme was active over a wide range of pH with optimum 11.0. Amit verma., et al., 2014 studied the keratinase enzyme production and found the optimum pH as
9.0 with maximum enzyme activity of 16.22 U/ml. The requirement of alkaline pH for optimum growth and protease production, clearly suggested the alkaliphilic nature of the organisms and keratinase enzyme.

Aida farag, et al., 2004; studied the optimum temperature of the free and immobilised enzyme, which showed the free enzyme had an optimum temperature of about 50°C, whereas that of immobilised enzyme shifted to 60°C. This optimum temperature for keratinase activity was in good agreement with other keratinase preparations obtained from Bacillus licheniformis., Lin, et al., 1992, Trichophyton scoloenleinii; Qin, et al., 1992, Streptomyces thermoviolacus, Chitte, et al., 1999 and Doratomyces microspores; Gradisar, et al., 2000. The effect of temperature on activity of the keratinase enzyme was investigated by Anbu, et al., 2005, the thermo labile nature of the enzyme, and the keratinase was stable at 25-50°C. However, the results are similar to keratinase from Streptomyces pactum and was active between 40 and 70°C (Bockle, et al., 1995); keratinase from D. microspores showed an optimal temperature at 50°C (Gradisar, et al., 2000).

Refai, et al., 2005 recorded the optimal reaction temperature for B. Pumilus FH9 keratinase is higher than those reported for B. pumilus (50°C) (Nitisinpraserts, et al., 1999). Many investigators reported optimum reaction temperature of 40-70°C for Keratinase enzyme from other Bacillus strains(Lin, et al., 1992, Lee, et al., 2002 and Gessesse, et al., 2003). Lonato, et al., 2008 studies showed the optimal temperature for activity was found in the range of 30-80°C for keratinsases production. San–Lang Wang, et al., 2008 studied the optimum temperatures for the three proteases namely P1, P2, P3 were 30-50°C, 40°C and 40-50°C respectively. Compared with Chryseobacterium sp. proteases, the optimum temperatures of three proteases are lower than C. indologenes IX9a protease (50°C) (Venter, et al., 1999) and Chryseobacterium sp. Kr6 Protease (50°C) (Riffel, et al., 2007) compared with Chryseobacterium sp. proteases.

Radhika Tatineni, et al., 2008 observed the keratinase production using Streptomyces sp. was 80% stable up to 50°C and completely inactivated at 70°C and higher temperatures. The enzyme is relatively thermo stable, with stability even after incubation at temperature of 50°C for 1 h. Bressollier, et al., 1999, Bockle, et al., 1995, Letourneau, et al., 1998 studied the production of keratinase
using *S. Pactum* DSM 40530, *Streptomyces SP.SK1-02*, *Scopulariopsis brevicaulis*, *B. licheniformis* and *K. rosea* and reached maximum production at 40 and 50°C. Geaun-Tae park, et al., 2009 have indicated the keratinolytic enzyme production using *B. Megaterium* F7-1 over a broader temperature range of 15-45°C than vibrio strain Kr2 and *Chryseobacterium strain Kr6* (Sangali and Brandelli, 2000; Riffel, et al., 2003).

Ana paula, et al., 2010 studied the keratinase production from *Bacillus* Sp. P7 possesses moderate thermo tolerance and thermo stability, which might be desirable features for the efficient control of enzyme reactivity in processes involving protein hydrolysis, as reported for neutral proteases. An increasing demand for keratinase production was observed particularly for alkaline proteases in the detergent industry due to its requirement for low temperature washing by maintaining on so the fabric quality which resulted in low energy needs (Venugopal and Saramma, 2006). Kumar, et al., 2011 reported the optimum temperature as 37°C for keratinase production by *B. altitudinis* GVC 11. Similar observations were reported with *B. licheniformis* RGI (Ramnani & Guptha, 2004), *Bacillus* sp. FK46 (Suntornsuk & Suntornsuk 2003). However, higher temperature optima of 40°C were reported for *B. subtilis KSI* (Suh, et al., 2001) and *Kocuria rosea LBP-3* (Bernal, et al., 2003) for production of keratinase and even more higher temperatures of 45°C and 70°C were recorded for isolates of *B. licheniformis*, (Yamamura, et al., 2002).

Thoomatti sudharsan Anita, et al., 2012 studied the keratinase enzyme production from *Aspergillus parasiticus* and the optimum temperature was found to be 50°C. Keratinases from fungi such as *D. Microsporus* (Gradisar, et al., 2000) and *Aspergillus oryzae*, (Farag, et al., 2004), also exhibited similar optimum temperatures. Venkata Saibabu, et al., 2013 used the isolated strain of *Bacillus megaterium* and showed that the isolate was capable of producing maximum keratinase production at 35°C. Further increase in temperature led to decline in enzyme production.
2.11 MEDIUM OPTIMIZATION

The concentrations of media components are really important as they are tools for bioprocess medium design (Çalık, et al., 2001). Culture medium supplies the microorganism with all the essential elements for microbial growth. Certain microorganisms are capable of synthesizing all of their cellular constituents from carbon and nitrogen sources. However most of the microorganisms require some source of micronutrients such as amino acids, trace elements, vitamins etc. The culture conditions that promote production of enzymes like proteases are significantly different from the culture conditions promoting cell growth (Moon & Parulekar, 1991). Therefore optimization of media component was required for optimum cell growth and product formation.

2.11.1 Effect of Carbon Source

Anbu, et al., 2007 studied the effect of carbon source on keratinase production using S. brevicaulis at 30°C and the maximum production was observed with 1% glucose as carbon source. The medium containing variety of sugars such as xylose, lactose, maltose, sucrose, sucrase and mannitol suppressed keratinase enzyme production. Suntornsuk and suntornsuk found that an addition of 1% glucose to medium containing feathers and yeast extract suppressed growth and keratinase synthesis of Bacillus sp. FK46. Gessesse, et al., 2003 showed the stimulation of keratinase production in the presence of glucose using B. pseudofirmus. Rammani and Gupta (2004) reported the optimization of medium for keratinase production using using B.subtilis RGI, and found that glucose was found to have positive effects. Santos et al., 1996 studied the keratinase enzyme production using Aspergillus fumigates was partially inhibited by glucose. Gousterova, et al., 2005 reported the maximum keratinase enzyme production utilizing wool substrate with the addition of starch using Streptomycyes flavis 2BG and Microblospora aerate IMBAS-IIA

2.11.2 Effect of Nitrogen Source

The nitrogen sources are of secondary energy sources for the organisms which play an important role in the growth of organism and the production. The nature and the composition of the compound used might stimulate or down modulate the production of the keratinase enzymes. Ramachandra, et al., 2011
reported that ammonium salts enhanced the enzyme activity and sodium nitrate showed a negative influence and a steep decrease in keratinase enzyme production was observed. Venkata Nagaraju, et al., 2013 studied the keratinase enzyme production under submerged fermentation and found that yeast extract was a better nitrogen source for the isolated strain of *Pseudomonas aeruginosa* with a maximum production of 90 U/ml. Refai, et al., 2005 reported neither ammonium chloride nor yeast extract and peptone showed a favourable effect on keratinase production using *B. pumilus FH9*. Wei-Hsun Lo, et al., 2012 reported the maximum keratinase production of 3.5 KU/ml with 2g/L of NH$_4$Cl as the nitrogen source after 54 h using *Bacillus cereus Wu2*. Johnvesly, et al., 2002 observed maximum production in the presence of 2% yeast extract on the first day of incubation. The maximum keratin hydrolyzing activity was achieved at higher yeast extract concentrations in a shorter period than in the presence of lower yeast extract concentrations. Because higher yeast extract concentrations provided higher concentrations of amino acids, proteins and vitamins that were essential for improved cell growth and production of keratinase enzyme.

### 2.11.3 Medium Optimization by Classical Method

Jin-Ha Jeong, et al., 2010 identified a novel feather-degrading *Xanthomonas sp. P5* and examined keratinolytic activity in improved medium containing 0.1% (w/v) feather and maximal keratinolytic activity was observed at 5 days (69.0 U/mL). This value was 7.1-fold highest than the yield in basal feather medium. The strain P5 degraded feather completely after 7 days. Feather degradation resulted in free thiol group, soluble protein and amino acids formation, indicating that sulfitolysis and proteolysis may be responsible for feather degradation by the strain P5. Total free amino acid concentration in the cell-free supernatant was around 188.6 mM. Asparagine, methionine, histidine and threonine were the major amino acids released in the culture. *Xanthomonas sp. P5* exhibited multiple plant growth-promoting attributes such as siderophore, indoleacetic acid, ammonia, hydrolytic enzyme and antifungal activity.

Musallam, et al., 2013 examined the degradation of feather using three strains of a novel thermophilic *Coprinopsis sp*. The strains grew in a mineral-based medium in which chicken feather was the sole source of carbon and nitrogen where they
decomposed the defatted and powdered feather meal into soluble amino acids fractions. Effective protease productivity was observed following 14, 21, 28 and 35 days of incubation and showed a gradual release of soluble amino acids into the medium, corresponding to a decrease in the dry weight of the substrate. The optimal extracellular cell-free keratinase activity was 32 KU/ml expressed in filtrates of 14-day-old cultures, and the optimal cell-bound keratinase activity was 39.9 KU/ml, expressed in centrifuged 21 day-old mats. All strains expressed optimal keratinase activities at 20.40°C and pH 9.

2.11.4 Medium Optimization by Plackett-Burmann Design and CCD

Cultivation conditions are essential in successful production of an enzyme, and optimization of parameters such as pH, temperature and media composition are important in developing the cultivation process. Despite all the work that has been done on production of proteolytic enzymes, relatively little information is available on Keratinases (Wang & Shih, 1999). The statistical approaches like Box-Wilson method and central composite design have been previously used by several researchers for media ingredient optimization. (Adinarayana, et al., 2002 & Rahman, et al., 2003). The optimization of some medium ingredient using the statistical techniques for production of keratinase using Bacillus subtilis with response surface methodology (RSM) for optimized production is an efficient way to design and predict the role of each ingredients in the resultant product.

Panchanathan Manivasagan, et al., 2013 reported the production of a thermo- and detergent stable keratinase using a marine actinobacterium belonging to the genus Actinoalloteichus from poultry feathers as a novel, inexpensive substrate. Medium composition and culture conditions for the keratinase production by Actinoalloteichus sp. MA-32 were optimized using two statistical methods: Plackett–Burman design was applied to find the key ingredients and conditions for the best yield of enzyme production and central composite design used to optimize the concentration of the five significant variables: whole chicken feather, soy flour, MgSO₄·7H₂O, KH₂PO₄ and NaCl. The medium optimization resulted in a 19.30-fold increase with a 31.99 % yield with a specific activity of 3842.57 U mg⁻¹ and the molecular weight was
estimated as 66 kDa. The enzyme was optimally active at pH 8–10 and temperature 50-60°C and it was most stable up to pH 12 and 10–14 % of NaCl concentration.

Manju, et al., 2013 described the isolated Bacillus subtilis showed the maximum keratinase activity in secondary screening using Casein agar medium and it was confirmed by 16s rRNA molecular sequence. The specific organism subjected to keratinase activity assay and identified by the spectrometric using the keratin azure as a substrate. Thus this organism was optimized by Response enzyme methodology (RSM) with the production medium. The optimum level of glucose was found to be 5.61g/l and the enzyme activity was around 13.280 U/ml. The keratinase production was favoured in the presence of ammonium tartarate as the nitrogen source; the enzyme activity in the presence of yeast extract (nitrogen source) was found to be 9.8±1.3 U/ml.

Long-Xian, et al., 2010 examined the medium optimization, purification, characterization and application of the keratinase from a newly isolated Chryseobacterium L99 sp. nov. were conducted. Exogenous sucrose, malt sugar, glucose, starch, tryptone, Mg\(^{2+}\), Zn\(^{2+}\), Ca\(^{2+}\) and Cu\(^{2+}\) could promote the keratinase production, while exogenous urea, NH\(_4\)Cl and yeast extract exhibited strong inhibition effects. Response surface methodology predicted a maximum keratinase yield of 213.8UmL\(^{-1}\), at (g L\(^{-1}\)) sucrose 16.8, MgCl\(_2\)·6H\(_2\)O 1.9, feather keratin 40.0, NaH\(_2\)PO\(_4\)·2H\(_2\)O 6.0 and K\(_2\)HPO\(_4\)·6H\(_2\)O 1.0, where dry cell weight nearly had a minimum 8.58 g L\(^{-1}\).

Jitendra Kumar, et al., 2012 described the five-level-five factors concept was utilized for the optimization for keratinase production by Acremonium strictum RKS1. Experiments were performed as a function Duration, pH, Amount of substrate, Nitrogen source and Carbon source. Optimization of these five parameters for the maximum production of keratinase was studied. Statistically designed experiment using response surface methodology was used to get more information about the significant effect and the interaction between the five parameters. 2\(^n\) full factorial central composite design was employed for the experimental design and analysis of result. The optimum process condition for maximum enzyme production are duration 12.8 days, pH 10, Amount of substrate 199mg, Nitrogen source 3.5% and Carbon
source 3%. The maximum keratinase production (92.7%) was achieved at the optimum process conditions.

2.12 OPTIMIZATION OF PROCESS PARAMETER

Matikeviciene, et al., 2011 identified and optimized significant technological parameters influencing keratinolytic enzyme production using *A. fradiae* 119 and to study its ability to degrade keratin. Chicken feathers meal (CFM) was found to be an excellent substrate for keratinase induction by *A. fradiae* 119. The strain produced 164 KU/mL keratinolytic activity in basal medium containing 7.5 g/L CFM as the sole source of carbon and nitrogen. Increased keratinolytic activity was achieved in media with ammonium sulfate as nitrogen source, the application of additional nitrogen sources to media containing CFM slightly decreased keratinase synthesis. Optimal parameters of the cultivation process were determined: pH of cultivation medium -7.2, temperature- 34°C and inoculum’s size 8 %, using the response surface methodology. The yield of keratinase activity was increased by 46% (267 KU/mL) after optimization of the cultivation process. The good ability of cultural liquid to degrade feathers and wool was detected.

Sivakumar, et al., 2012, isolated the keratinase producing bacterial strains from the chicken feather dumping site and the bacterial strains were identified by physical, biochemical characteristics, fatty acid methyl ester (FAME) and 16S r DNA sequences. Medium optimization of the selected keratinase producing bacterial strain (*Bacillus thuringiensis* TS2) was checked with chicken feather as substrate and various parameters such as incubation time (52.3±1.2 U/mg), substrate concentration (41.3±1.2 U/mg), inoculums concentration (43.54±0.89 U/mg), carbon sources (76.20±0.34U/mg), nitrogen sources(85.60±3.12U/mg), pH(90.78±0.97U/mg), temperature(92.78±0.59U/mg) and chemical inhibitors (112.17±0.02U/mg) were tested with specific keratinase activity.

Sivakumar, et al., 2011 reported the optimum parameters for keratinase production using *Bacillus cereus* TS1 with response surface methodology (RSM) based on central composite design (CCD) model. Statistical testing was performed for analysis of variance (ANOVA) for quadratic regression equations of both linear and interaction effect of variables. Optimum conditions for keratinase production
using *Bacillus cereus* were found to be pH 9, temperature 50°C and starch-1% and the maximum keratinase production observed by the model was 60.67 U/ml.

### 2.13 PRODUCTION OF KERATINASE ENZYME

Sandhya, et al., 2005 studied the keratinase enzyme production both in submerged fermentation (SmF) and solid-state fermentation (SSF). Both of them influence various aspects of the growth of the microorganism as well as enzyme production. Jian-Fa ou, *et al.*, 2012 investigation showed that the SmFs are usually carried out with a dissolved or suspended substrate in an aqueous medium. Previous experimentation by Vierheller, *et al.*, 1995 clearly indicated that many types of submerged fermentations have been described of which continuous cultures have proven to yield better enzyme production than batch fermentations. Prakasham, *et al.*, 2006 reported the cost of production medium used for submerged fermentation was considerably high and solid-state fermentation has gained renewed interest over submerged fermentation due to the recent developments in solid waste treatment and the production of secondary metabolites.

Ekta Tiwary, *et al.*, 2010 reported a novel dimeric 58 kDa keratinase from *Bacillus licheniformis ER-15*. The bacterium produced 244 U/ml keratinase in 48 h which was increased by eight fold (1962 U/ml) after medium optimization by one-variable-at-a-time and response surface methodology. Enzyme was concentrated by ultrafiltration followed by acetone precipitation and purified by gel filtration chromatography. It had subunit of 30 and 28 kDa. Enzyme was maximally active at pH 11 and 70°C hydrolyzed various complex proteins viz. haemoglobin, feather, hooves, fibrin and meat protein. It was a thiol activated serine protease and 6.25-fold enhancement in activity was observed in presence of 5 mM mercapto ethanol. Nearly 1200 U keratinase degraded 1.5 g feather in 12 h at pH 8, 50°C in redox free environment. This enzyme also dehaired buffalo hide within 16 h in presence of 3% Ca (OH)$_2$. Fig. 2.13.1 gives a flowsheet for the production of keratinase enzyme by microbial fermentation technique. Table.2.13.1 gives a summary of various microorganisms used for keratinase production.
Identification/Isolation of the strain of the microorganism

Preservation of the strain

Culturing / growing of inoculums

Pre-fermentation culturing

Fermentation process

Aerobic

Anaerobic

Shake

Culturing of spores on solid medium

Batch

Continuous

Fed batch

Removal of biomass

Primary isolation of the product

Cell disruption

Physical

Solid shear

Agitation with abrasion

Freezing-Thawing

Detergent

Osmotic shock

Alkali treatment

Enzyme

Precipitation

Filtration

Centrifugation

Liquid-Liquid

Chromatography

Ultrafiltration

Drying

Crystallization

Physical

Chemical

Biological

Treatment of effluents

Recovery and purification of the product

Product Isolation and purification

Fig. 2.13.1 Keratinase Enzyme Production by Fermentation
<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Substrate</th>
<th>Medium Composition (g/L)</th>
<th>Operating conditions</th>
<th>Fermentation type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>Chicken feather</td>
<td>NH₄Cl-0.5, NaCl-0.5, K₃HPO₄-0.3, KH₂PO₄-0.3, MgCl.6H₂O-0.1, Yeast extract-0.1, Feather-10</td>
<td>6-9</td>
<td>40-70</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Kocuria rosea</em></td>
<td>Chicken feather</td>
<td>Yeast extract-0, Milled feather-20</td>
<td>3-11</td>
<td>10-90</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Chryseobacterium Sp.Kr6 Strain</em></td>
<td>Whole Poultry feather</td>
<td>NaCl-0.5, K₃HPO₄-0.3, KH₂PO₄-0.4 Whole Chicken feather-10</td>
<td>6-10</td>
<td>30-100</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Chryseobacterium Indologenes TKU014</em></td>
<td>Squid pen powder Shrimp shell powder Chicken feather</td>
<td>SSP-0.5%, K₃HPO₄-0.1%, MgSO₄·7H₂O-0.05%</td>
<td>7-10</td>
<td>30-50</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Trichophyton Sp.HA-2</em></td>
<td>Feather</td>
<td>Dextrose-40, Peptone-10, Agar-20</td>
<td>4-10</td>
<td>20-50</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Bacillus Megaterium F7-1</em></td>
<td>Chicken Feather meal</td>
<td>NH₄Cl-0.5, NaCl-0.5, K₃HPO₄-0.2 KH₂PO₄-0.1, MgCl.6H₂O-0.1, Yeast extract-0.1, Feather-10</td>
<td>4-11</td>
<td>10-45</td>
<td>Submerged</td>
</tr>
<tr>
<td><em>Bacillus Pumilus</em></td>
<td>Feather and human hair</td>
<td>KH₂PO₄-0.7, K₃HPO₄-1.4, NaCl-0.5, MgSO₄·7H₂O-0.1</td>
<td>6-9</td>
<td>45-60</td>
<td>Submerged</td>
</tr>
</tbody>
</table>

Table 2.13.1 Production of keratinase enzyme using various microorganisms utilising chicken feather as substrate
<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Substrate</th>
<th>Medium Composition (g/L)</th>
<th>Operating conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus Sp.p</strong></td>
<td>Feather</td>
<td>Whole chicken feather-5, Peptone-0.5, Glucose-1, KH$_2$PO$_4$-0.1, K$_2$HPO$_4$-0.3</td>
<td>5-12</td>
<td>Ana Pale et al., 2010</td>
</tr>
<tr>
<td><strong>Bacillus Licheniformis ER-15</strong></td>
<td>Chicken feather</td>
<td>Feather-10, Yeast extract-0.1, MgSO$_4$.7H$_2$O-0.1, NH$_4$Cl-0.5, K$_2$HPO$_4$-0.3</td>
<td>7-12</td>
<td>Thota Tiwary et al., 2010</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>Feather and Keratin</td>
<td>KH$_2$PO$_4$-1.5, K$_2$HPO$_4$-1.0, MgSO$_4$.7H$_2$O-0.2, CaCl$_2$.2H$_2$O-0.2, NaCl-0.2, ZnSO$_4$.7H$_2$O-0.003, Yeast extract-0.1, Glucose-6, Glycerol-2ml</td>
<td>7-9</td>
<td>S. M. Harde et al., 2011</td>
</tr>
<tr>
<td><strong>Aspergillus parasiticus</strong></td>
<td>Chicken feather</td>
<td>Starch, Casein, Agar</td>
<td>4-9</td>
<td>Thoomatti Sudharsan et al., 2012</td>
</tr>
<tr>
<td><strong>Streptomyces albus</strong></td>
<td>Chicken feather</td>
<td>Starch, Casein, Agar</td>
<td>4-11</td>
<td>Srinivasan Nayaka et al., 2013</td>
</tr>
</tbody>
</table>
2.13.1 Fermentation and Other Microbial Processes

Austin, 1984 survey showed that fermentation is the process of anaerobic breakdown or fragmentation of organic compounds by the metabolic processes of microorganisms. However, fermentation processes can be considered generally to relate to the chemical changes of a substrate accomplished by selected microorganisms or extracts of microorganisms to yield a useful product. This less specific definition includes microbiological processes carried out under anaerobic, aerobic and enzymatic conditions.

Most importantly a fermentation process is very often the only feasible way to obtain a complex, or a highly specific chemical transformation of the raw material. Also a chemical change conducted enzymatically has a far lower energy barrier incurred by the process than a chemical synthesis route to the same change. This was because the process may very often be carried out enzymatically at ambient or near ambient conditions. Under these conditions the enzymatic processes can be $10^9$ to $10^{12}$ times as rapid as the corresponding chemical route to the same product. Hocken-hull was credited with the quotation that “once a fermentation has been stated it can be made worse but not better” (Calam, 1976). Whereas this is an over statement it does illustrate the importance of inoculum development, much of the variation observed in small scale laboratory fermentation is due to poor inoculum being used and thus it was essential to appreciate that the establishment of an effective inoculum development programme was equally important regardless of the scale of fermentation.

2.13.2 Production of Keratinase Enzyme by Solid State Fermentation

Mitchell and Lonsane, 1990 and Pandey, et al., 2001 state that, solid-state fermentation (SSF) is the fermentation involving solids, substrate must possess enough moisture to support growth and metabolism of microorganism. Suggestions by Pandey, et al., 2000 showed that filamentous fungi are the best studied for SSF of keratinase enzyme due to their hyphal growth, which have the capability to not only grow on the surface of the substrate particles but also penetrate through the particles. Williams, et al., 1990 studied the solid state
fermentation techniques using feather degrading microbes (*Bacillus subtilis*) was grown aerobically to a concentration of approximately $10^7$ cell/ml in the chicken feather broth containing 1(%w/v) of raw chicken feather and achieved maximum keratinase production of 1456.10 KU/ml. Aliaa Borai, et al., 2013, produced keratinase using *A. terreus* cultures in solid state fermentation utilising coconut powder as a substrate. Similarly Sandhya, et al., 2005 carried out solid state fermentation and utilized various agro industrial residues such as wheat bran, rice bran, coconut oil cake, sesame oil cake and olive oil cake for neutral protease production using *Aspergillus oryzae 1808*. Rai, et al., 2009 experimentation showed a significant increase in keratinase production using *Bacillus subtilis RM-01* and chicken feather supplemented with maltose and sodium nitrite as a substrate.

Sudhir Rai, et al., 2009 reported the application of response surface methodology (RSM) for optimization of the media composition for keratinase production by a feather degrading *Bacillus subtilis* strain RM-01 in solid-state fermentation using chicken-feather as substrate. The optimized culture conditions with 5.0 g of chicken feathers moistened with distilled water (1:1, w/v, adjusted to pH 8.0) supplemented with maltose (10%, w/w) and sodium nitrate (1.25%, w/w) as best co-carbon and co-nitrogen sources respectively resulted in 5-fold increase of keratinase production post-72 h of incubation at 50°C compared to keratinase production under un-optimized condition.

Mona Esawy, 2007 used different substrates as the sole carbon and nitrogen source for keratinase production under solid state fermentation conditions (SSF). Maximum keratinase production was achieved (1103 U/g solid substrate) with wheat flour 10 g/flask at 150% moisture content after 5 days at 30°C. The partially purified enzyme showed its maximum activity at 65°C, pH 10 and complete stability after one hour incubation at 70°C. The metal ions such as Zn$^{2+}$ and Ca$^{2+}$ played a significant role in enzyme stability. This keratinolytic enzyme offers an interesting potential for the hydrolysis of different keratin wastes to be used as feed supplement or bioconversion to added-value products. The enzyme is unique in that unlike most other microbial serine proteases
known so far, it did not show hydrolytic activity towards casein and BSA. Microscopic analysis showed complete lyses of the native cow horn particles.

Ponnuswamy Vijayaraghavan, et al., 2012 used agro-industrial residues for the production of a halotolerant keratinolytic - protease using *Actinobacterium* sp. in solid-state fermentation. Among various agro-industrial residues that were evaluated, apple pomace supported maximum protease production (8400 U/g material). The optimum conditions required for enzyme production were a fermentation period of 72 h, 10% (w/v) NaCl, pH 7.0, 120% (v/w) moisture and 10% (v/w) inoculum. The enzyme exhibited activity to a range of pH (7.0-9.0) and temperature (30-45°C), with optima at 8.0 and 40°C respectively. Most of the divalent ions tested stimulated the protease activity and Ca\(^{2+}\) ion was required for its activity and stability. The enzyme was widely active at the range of NaCl concentration (5%-15%, w/v) and effectively degraded chicken feather. This protease could be useful in fish sauce fermentation and also in feed industry.

### 2.13.3 Production of Keratinase Enzyme by Submerged Fermentation

Friedrich, et al., 1990 have utilized the keratin powder (sterile chicken feather were ground) was taken directly to the fermentation medium. The powder was shifted through a 0.5 mm size. The submerged fermentation was followed according to the method of Nickerson, et al., 1963. The *Bacillus subtilis* showed a maximum keratinase production of 18000 KU/ml at 48 h with the specific pH 8.0 and temperature 50°C.

Venkata Naga Raju, et al., 2013 utilized the isolated strain of *Pseudomonas aeruginosa* from poultry waste and tested its abilities to hydrolyze the feather. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source (Organic and inorganic) incubation time, Inoculum sizes and surfactants on keratinase production was studied using isolated bacterial strain under submerged fermentation (SmF) condition. The maximum keratinase production were observed with maltose (120±2.6 U/ml), yeast extract (90±3.6 U/ml), ammonium sulphate (61±3.0 U/ml), pH 7.0 (114±4.1), temperature 40°C (110±1.9), Tween-80 (104±2.8U/ml), inoculum size level 5% (112±2.0U/ml) and incubation time 48 hours (101.01±0.56U/ml) in the production medium.
Tappa Mohammad Munawar, et al., 2012 experimentation showed that *Bacillus licheniformis* was able to produce keratinase for the substrates feathers, feather meal and human hair. Basal medium was prepared and inoculated with the strain and incubated for 7 days at room temperature on a rotatory shaker at 100 rpm. Crude enzyme was obtained and it was assayed for keratinolytic activity and degree of feather degradation. Among the three keratin substrates employed maximum enzyme was produced from feather meal. The degree of degradation was also found to be maximum in feather meal. Thus inferred that feather meal produced the maximum amount of enzyme compared to other substrates.

Deivasigamani, et al., 2008 used isolated *Bacillus sp.* from slaughter house and poultry farm area and observed maximum keratinase activity of (122.5 KU ml-1) with azokeratin medium at pH 8.0. Submerged fermentation was carried out at pH 8.0 for 5 days. On 4th day the enzyme production was highest (140 KU ml-1) with 1% feather (w/v). Crude protein (75%) was obtained and measured in terms of 1.44 mg ml$^{-1}$ on fifth day. The molecular weight of this keratinase was 32 kDa by SDS-PAGE. The crude protein from feather has of high nutrient value and could be used as animal feed for livestock and fish feed in aquaculture.

Masoomeh Shams Ghahfarokhi, et al., 2003 state that the maximum keratinolytic activity with submerged cultivation was reported for cultures of *T. mentagrophytes* isolate No. 1 grown for a 12-day period at 32°C. Extracellular keratinase activity was in the range of 0.28 to 2.18 u/mg protein in different isolates at predetermined optimal conditions. The growth of *T. mentagrophytes* isolate No. 1 was inhibited in the presence of various concentrations of onion and garlic extracts. This inhibition reached to a maximum of 100% for both extracts at 10% v/v concentrations. Keratinase synthesis was also inhibited by two extracts as a dose-dependent manner with maximums about 58.54 and 71.36 percent at 5% concentrations, accordingly. In contrast to the fungal growth keratinolytic activity was inhibited more by garlic as compared with onion extract.

Kanchana, 2012 utilized the optimized culture medium for the production of extra cellular keratinase using a newly isolated strain of *Bacillus sp.* in shake-flask culture. The keratinase production was increased by approximately 40-fold when the
strain was grown in an inexpensive optimized medium (120 U.mL\(^{-1}\)) compared to the un-optimized medium (3.80 U.mL\(^{-1}\)). The maximum amount of keratinase activity was produced at 37°C and the bacterium were cultured for 72 h in medium containing feather meal as the sole source of carbon and 0.025% yeast extract with initial pH of 7.0 under submerged fermentation. The isolated keratinilytic *Bacillus* sp also exhibited remarkable feather degrading ability.

### 2.13.4 Production of Keratinase Enzyme in a Fermentor

Yoon-jeong lee, et al., 2004 investigated the effects of cultivation modes on keratinase production by flask-shaking culture and batch culture using a jar fermentor (7 L) under the optimum conditions. Enzyme production in the flask culture (stationary phase) increased to maximum levels of cell growth and maximum productivity after 84h and its keratinase activity was measured at 90 U/ml. However the maximum cell growth and keratinase production in the batch culture was reached after 24 h and 48 h (25 U/ml) respectively. Even though growth and keratinase production were faster in the batch culture with the fermentor than those of the flask culture, keratinase production of the batch culture was about 4 folds lower than that of the flask culture. From the results of protein analysis of fermentation broth after fermentation, it was probably due to more faster exhaustion of keratin in the fermentor culture than in the flask culture. Therefore the flask culture was considered to be good for mass production of keratinase from *paracoccus sp. WJ-98*. Chon, et al., 2000 pointed out the productivity of keratinase 360 UmL\(^{-1}\)h\(^{-1}\) was about 3 folds lower than that of *Pseudomonas sp. Kp-364* (1,270 Um L\(^{-1}\)h\(^{-1}\)).

Rinky Rajput, et al., 2013 reported the production of thermostable keratinase using *Bacillus pumilus KS12* was enhanced up to seven fold by statistical methods. The enzyme was partially purified by ultra filtration followed by thermal precipitation with purity of 3.2-fold and recovery of 89%. Keratinase was immobilized using covalent method by cross linking 2 mg protein (688 U/mg) on to 1 g chitin activated with 2.5% (v/v) glutaraldehyde for 60 min. Its comparative biochemical studies with that of free keratinase revealed that the shift in optimum pH with increased stability towards pH from 9.0 to 10.0 and
temperature. Also, it showed statistically significant improved hydrolysis of a number of soluble and insoluble substrates in comparison to free keratinase. Owing to improved catalytic efficiency of immobilized keratinase, its potential for degradation of Sup35NM were evaluated, where 100 µg of enzyme could degrade 60 µg Sup35NM after 60 min at pH 7.0 and 37 °C.

2.13.5 Production of Keratinase Enzyme by Immobilization

Susmita, et al., 2012 studied the immobilization of keratinase in calcium alginate gel was the most favourable, since the percent entrapped activity was maximum in calcium alginate beads 45.77%. One bead having an one µg of the keratin. These beads undergone with the retention time for immobilized beads increase from 2 to 5 hours. The activity of the calcium alginate entrapped keratinase beads was assayed three cycle using the substrate. The above beads displayed high level of heat stability and increased tolerance towards acidic pH compared with the free keratinase. These beads are stored in IXPO4 buffer, and retained its 50% of the original enzyme activity after 7 days. It can be easily stored, filtered from the fermented broth taken, maintain activity, in-contaminated for long life.

Aliaa Borai, et al., 2013 reported the production of extracellular keratinase using locally isolated Aspergillus terreus and medium optimization was carried out using Plackett-Burman design, under optimized media by statistical design. The keratinase activity reached about 1.63 times higher than that obtained from the control medium. The best solid substrate for keratinase activity was coconut powder. Alginate with 2.5 ml volume was the best immobilization gel materials for keratinase activity. Adsorption of fungal mycelia on clay particles showed high keratinase activity (0.314 U/ml) and protein content, however lower than that of free cells. Reusing entrapped and adsorbed cultures of A. terreus led to a gradual decrease of keratinase activity in the cultures of the 2nd run and continued to decrease up to the 5th run.

2.14 PURIFICATION OF KERATINASES

In addition to the higher keratinase production under optimal conditions, purification of keratinase is necessary for further industrial applications to
hasten the efficiency of keratinase action. 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 200 kDa from different strains of bacteria and fungi. However, Kim et al., 2004 reported recovery of keratinase with a molecular weight of 440 kDa. 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. Using a similar strategy, Zhang, et al., 2009 purified the alkaline keratinase from *Bacillus* species and identified keratinase of 27 kDa using MALDI-TOF-MS. Anbu, et al., 2005 isolated keratinase with a molecular weight of 39 kDa from the poultry farm isolate, *Scopulariopsis brevicaulis*, and found that this keratinase had a serine residue near the active site.

2.15 KINETIC MODEL

The modeling of enzyme production kinetics is very difficult and challenging job for model development, i.e., model formulation, identification and estimation of the parameters, and solution of the equations (Coppella & Dhurjati, 1989).

2.15.1 Classification of Models

Many classes of models can be distinguished for microbial kinetics. In general, fermentation process models are either structured or unstructured depending on the metabolic analysis pattern. Structured models use the information on intracellular metabolic pathway reaction rates, Structured models describe culture response to large or rapid perturbations satisfactorily. Structured models deals with the inner structure of cells and thus acknowledge only implicitly the changes of cellular physiological state with the environment. The unstructured models are relatively easy to build and are used extensively in simulating steady state or slowly changing systems (Amrane & Prigent, 1999; Weiss & Ollis, 1980).

Structured models are superior to unstructured models because they can provide a measure of the quality of the cell population. These models incorporate biological knowledge by separating the biomaterial into compartments. The interactions between compartment–compartment and compartment-environment are
described by equations on stoichiometry and kinetics especially on mass and energy balances, on the rates of transport and on reactions (Thilakavathi, et al., 2007). Structured models can be used in a broader domain of conditions and have better capacity to extrapolate than unstructured ones (Kim & Shuler, 1990; Lee & Bailey, 1984; Nielsen, et al., 1989).

### 2.15.2 Unstructured Model

The unstructured models utilize the knowledge of experimental reality and describe biomass and associated metabolite production. The unstructured model presumes the balanced growth which is equivalent to fixed cell composition. Such assumption is valid primarily in single-stage, steady-state continuous culture and the exponential phase of batch culture and it fails to discuss during any transient condition. If cell response is fast then external changes and if the magnitude of these changes is not too large, the use of unstructured models can be used because the deviation from balanced growth may be small.

Unstructured kinetic models are the most frequently employed for modelling microbial systems based on simplicity and technical robustness. However, the formulation of an unstructured model was based on Monod model and logistics model (Esener, et al., 1983; Shuler & Kargi, 2008). Unstructured models are simpler than the structured models. Thus it is necessary to measure smaller numbers of important components. So the model may describe at least the evolution of biomass; carbon sources; products; and often, another substrate, mainly nitrogen (Moraine & Rogovin, 1971). In a typical fermentation process, a number of these rate processes are important, viz, the rate of change of the amount of biomass and its different components, the rate of consumption of nutrients, and the rate of production of products and metabolites (Thilakavathi, et al., 2007).

In general, the biomass growth is modeled using Monod’s equation or logistics equation for the specific growth of cells. The coefficient of the microbial decay for the microbial growth is also included in the differential equation (Moraine & Rogovin, 1971). Usually substrate consumption is explained with the equation of either biomass growth and yield coefficient for biomass production or product production and its yield coefficient (Amrane & Prigent, 1994). The
differential equation concerning product concentration was explained using the Luedeking and Piret equation (Luedeking & Piret, 2000).

### 2.15.3 Kinetic Models for Bacterial growth Kinetics

Muhammad Nadeem, et al., 2009 studied the production of alkaline protease using *B. licheniformis N-2* in shake flask experiments with growth medium comprised of (g/l): glucose, 10; soybean meal, 10; K₂HPO₄, 5; MgSO₄.7H₂O, 0.5; NaCl, 0.5; CaCl₂.2H₂O, 0.5. Different process parameters such as fermentation period, initial pH, incubation temperature, agitation speed and size and age of inoculums were optimized for the maximum yield of the alkaline protease. Maximum enzyme production (991.04 PU/ml) was obtained after 24 h of incubation at 37°C in growth medium of pH 10. The optimum inoculum size and age were determined as 2.0% (v/v) and 24 h, respectively. Kinetics of growth and protease production by this microorganism were also analyzed at all optimum conditions.

Biomass (Yₓ/s) and product yield (Yᵧ/s) coefficients were determined as 0.375 g of cells/g of glucose and 99.3 PU/g of glucose with a growth rate of (0.215 h⁻¹) after 24 h of incubation. Thereafter a continuous decrease in biomass and product yield was observed. The results indicate that enzyme production by *B. licheniformis N-2* was entirely growth-associated and that a major portion of the enzyme was secreted in the post exponential phase.