CHAPTER – I

INTRODUCTION

1.1 INDUSTRIAL ENZYMES

“All enzymes are proteins but all proteins are not enzymes” Proteolytic enzymes account for nearly 60% of the industrial enzyme market. Proteases of commercial importance are produced from microbial, animal and plant sources. The proteases constitute a very large and complex group of enzymes which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Commercial proteases have application in a range of processes which take advantage of the unique physical and catalytic properties of individual proteolytic enzyme types particularly in detergent, leather, food and pharmaceutical industries. (Rao, et al., 1998). Milk clotting enzymes have been used to transform milk into products such as cheese since about 5000 BC, when it was observed that milk carried in calf stomachs tended to curdle. Pancreatic proteases were used for dehairing and bating of hides and as presoak detergents since about 1910.

Major interest in commercial detergent proteases developed in the 1950s and enzyme detergents captured a substantial share of the detergent market during the 1960s. In 1969 the industry met with unfavourable publicity when some detergent workers developed allergies due to the dusting effects of the enzymes. The problem was overcome by the introduction of dust free preparations for use in detergents. Alkaline detergent proteases now account for 25% of the total industrial enzyme sales. Protease have a wide range of functions in nature. Extracellular microbial protease contribute to the nutritional well being of the producing organism by hydrolyzing large polypeptides substrates into smaller molecules that the cell can absorb. Microbial proteases are produced from high yielding strains by fermentation under controlled conditions in surface or submerged culture. The enzymes are produced extracellularly and recovery involves separation of cell free liquor by filtration or centrifugation.
Depending on the product and the degree of purity required, further purification might involve steps such as concentration, precipitation and stabilization. Production of enzymes from plant or animal sources usually involves grinding or mincing of the material followed by a series of extraction and purification steps to achieve a product of stable and standardized activity (Ward). Wastes of animal origin such as nails, feathers, horns, hair and other hard-to-degrade keratin containing materials from poultry and cattle slaughter houses, comprising keratins can be processed and converted to useful products by the action of special class of proteases, keratinases (Onifade, et al., 1998). The market for keratinase enzyme is less now when compared to other industrial enzymes, and the demand is growing day by day.

The cheaper materials as substrate were tried for economic production by Holker, et al., 2005; Das, et al., 2007 and Mukherjee, et al., 2008). Keratinase is an extracellular enzyme used for the biodegradation of keratin and is produced only in the presence of keratin substrate. Microbes which produce Keratinases are proved to metabolize various substrate sources of powdered chicken feather, hair, nail, wool etc (Gradisar, 2005; Cai, et al., 2009). This enzyme was produced by Bacillus licheniformis (Rammani, et al., 2005; Korkmaz, et al., 2004; Williams, et al., 1990; Zerdani, et al., 2004), Burkholderia sp, Pseudomonas sp, Microbacterium sp (Bernal, et al., 2006).

A number of keratinolytic microorganisms have been reported to produce keratinase from fungi such as Microsporum. sp (Anbu, et al., 2008) and from the bacteria Bacillus and Streptomyces sp (Cai, et al., 2009; Macedo, et al., 2005). Optimization of medium components is one of the most important parameters by which the enzyme production cost could be reduced by 30-40 % (Kirk, et al., 2002). The classical method is used to study one variable at a time keeping other variables constant. This method is very much time consuming, expensive and does not reflect true optimum when a large number of variables are studied. The interactive effects between the variables are not included in this method (Haji, M. et al., 2008). The Placket-Burman Design is used to screen the medium components with respect to their main effects and not their interaction effects on maximum enzyme production (Placket and Burman., 1946). The Placket-Burman Design is an effective and efficient technique
for the optimization of medium components and can be used to select the significant factors and to eliminate the insignificant one in order to obtain more manageable and smaller set of factors. Based on Plackett-Burman Design, each factor is examined at two levels, low (-1) and high (+1) (Jalbani, et al., 2006). Design of experiments (DOE) is a systematic rigorous approach to engineering problem solving that applies principles and techniques at the data collection stage so as to ensure the generation of valid, defensible and supportable engineering conclusions. In addition all of this is carried out under the constraint of a minimal expenditure of engineering runs, time and money. The objective is to predict the response values for all possible combinations of factors within the experimental region and to identify an optimal experimental point (Bandaru, et al., 2006). Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the aim is to optimize this response. The individual effect, interactive effect and squared effects could be assessed (Reddy, et al., 2008; Hujanen, et al., 2001; Rao, et al., 2007; Ghosalkar, et al., 2008; De Coninck, et al., 2000). In the present research work the production of keratinolytic thermostable enzyme using chicken feather powder, goose feather powder and rice husk as substrates was attempted using *B. subtilis* and *S. griseus*.

1.2 CLASSIFICATION OF PROTEOLYTIC ENZYMES

Proteolytic enzymes are ubiquitous in terms of occurrence; they are found in all living organisms, and are essential for cell growth. Although protease production is an inherent feature of the earth biota, very few proteases were exploited for commercial production particularly using *Bacillus* species (Gupta et al., 2002b; Fujinami & Fujisawa, 2010; Rao et al., 1998). Currently proteases are classified on the following major criteria.

1. Type of reaction catalysed
2. Chemical nature of the catalytic site
3. Evolutionary relationship with reference to structure.

Keratinase belongs to a class of hydrolases that can hydrolyze peptide bonds, thus forming a distinct two sets of subclasses of peptidases such as exopeptidases (EC 3.4. 11-19), which act only near either terminus of a
polypeptide chain; and endopeptidases (EC 3.4.21-24 and EC 3.4.99), which act preferentially away from those termini. Hence peptidases are not only classified based on the reactions they catalyse, but also on the catalytic type. As a consequence different proteins can be regarded as distinct peptidases with similar or identical peptidase activities (Beynon & Bond 2001).

1.3 KERATINASES

Keratinases was first coined by Kuhne in 1878 and it was analysed by Pesox in 1883. Basically, there are keratin observed in the hair, hooves of mammals, horn, claw and β-helix keratin are present in the claw of reptiles, nail and scales. α-helix keratins are packed as folded with cysteine. Based on the percentage of sulphur content, keratins are classified into hard and soft keratin. Hard keratin comprises of 5% sulphur with low lipid content and thermal stability is high vice versa the soft keratin having 1% sulphur, lipid stability is low. (Manju, 2013). Keratinase hydrolyze insoluble keratins more efficient than other proteases (Vighneshwaran, et al., 2010). Keratinases have broad substrate specificity and are more active against both soluble and insoluble keratin containing substrates. Among soluble proteins, they possess the ability to hydrolyze haemoglobin, casein, bovine serum albumin and gelatin whereas among insoluble proteins, they hydrolyze, wool, silk, elastin, horn stratum cornea, collagen hair, azokeratin, feathers and nail (Bressollier, et al., 1999).

The tight structural arrangement of keratin hinders the proteolytic access to its cleavage sites such that it is degraded mainly by specific class of proteases called keratinases. The structural configuration of keratinases enables the interaction with β-keratin and assist in disassembling of the layers of β-structures so that individual strands can be hydrolysed subsequently (Kim, et al., 2004).

1.4 APPLICATIONS OF KERATINASES

The potential industrial applications of keratinases are summarised in Table 1.4.1 (Brandelli, et al., 2010)

Table 1.5.1 Industrial Applications of Microbial Keratinases
Prion diseases are a group of progressive condition that affect the brain and nervous system. Misshaped prion production carries the disease between individuals and cause deterioration of the brain. It cannot spread through the air or through touching or casual contact but through contact with infected tissue, body fluids or contaminated medical instruments (Shimomura, et al., 2003). Bacterial keratinases produced by *B. licheniformis* strain *PWD* 1 degrade prion protein (PrPC) in brain, stem tissue from animals with bovine spongiiform encephalopathy and scrapie degradation of surrogate yeast prion protein. Prion proteins are causative agents of transmissible spongiform encephalopathy a serious neurodegenerative diseases. (Narasimha murthy, et al., 2007)


The treatment of nail disease is limited by the low permeability of drugs through the nail plate. Keratinolytic enzymes might decrease the barrier properties of the nail plate and increase drug diffusion by hydrolysing the nail
keratins. Then the addition of a reducing agent (Dithiothreitol, DTT) would increase the damage caused to the nail. So the enzymatic disruption of nail plate is translated into enhanced drug penetration, into the nail plate (Rani Gupta et al., 2006). The permeability and partition co-efficient and the drug reflux are found to be appreciably increased in the presence of the enzyme (Robert Preston, 2001).

Dermatophytosis or ringworm is a clinical condition caused by fungal infection of the skin in humans and tame animals such as sheep, cat and cattle. The fungi that cause parasitic infection (dermatophytes) feed on keratin and is present in the epidermis of hair, skin and nails. Dermatophytes commonly gain entry into the host via (keratin rich hair, skin or nails. Thus keratinolytic protease (keratinase) is currently used in the treatment of dermatophytosis (Xiang lin, et al., 1992), (Ramadharkumar, et al., 2010).

Keratinase synthesized from Bacillus licheniformis (PWD) and E.Coli has been adapted to the laboratory and cosmetic applications especially acne treatment (Subhasish Saha, et al., 2010). Keratinase is included in facial lotions, and it is useful for treating acne, because dead cells can clog pores and create a favourable environment for acne formation. Shampoos for anti–dandruff include keratinase, because the keratinolytic action allows shampoo to clean flaky dead cells and can remove warts, calluses and corns. The U.S food and drug administration (FDA) has approved keratinase for curing psoriasis, a condition involving excessive turnover of skin cells and scaly build up (Veslava Matikevicience et al., 2009).

1.5 NEED FOR THE PRESENT STUDY

Since keratinase has a vast demand due to variety of applications attempts are made to produce keratinase enzyme efficiently and economically. Fermentation process gives promising yield of enzymes and is an economical method due to low costs and accessibility of waste materials as substrates. Presently, efforts are being made to increase the production of keratinolytic enzymes by fermenting the poultry and agricultural waste materials through biotechnological approaches. Poultry industries generates huge quantum of poultry residues which are difficult to dispose off, and their use as substrates
for keratinase production not only reduce the production cost of enzyme but also combat environmental pollution. The disposal of poultry waste and the agricultural waste creates pollution.

1.6 OBJECTIVES OF THE PRESENT STUDY

The objectives of the present research work are given below:

1. To study the keratinase enzyme production by *Bacillus subtilis* (NCIM 2724) and *Streptomyces griseus* (NCIM 2622) under SmF (Submerged fermentation).

2. To investigate the effects of various substrates such as chicken feather powder, goose feather powder and rice husk on keratinase production using the above two microorganisms.

3. To study the effects of process variables on keratinase enzyme production by classical method.

4. To screen the most significant medium components that affect Keratinase production using Placket- Burman Design and identify the most significant components.

5. Optimization of the most significant variables by Central Composite Design (CCD) and Response Surface Methodology (RSM) using *B. subtilis* and *S. griseus*.

6. Optimisation of the process parameters such as incubation period, temperature, initial pH, substrate concentration, and inoculum size for the maximum production of keratinase enzyme by RSM using *B. subtilis* and *S. griseus* with CFP, GFP and RH as substrates.

7. Experimental validation of the second order polynomial model of the RSM.

8. To study the kinetics of keratinase enzyme production under optimum conditions using the optimized media using *B. subtilis* and *S. griseus* with CFP as substrates.

1.7 ORGANIZATION OF THE THESIS

Chapter 1 – “Introduction” deals with the definition, classification and industrial importance of keratinase enzyme. It also explains the need for carrying out the present research work.

Chapter 2 – “Literature Review” includes a detailed literature about the various sources of keratinase enzyme production, characterization and industrial applications of keratinase enzyme. This also covers the production of keratinase enzyme by Bacillus and Streptomyces sp., by submerged fermentation. This covers the conventional and statistical methods of optimization for keratinase production by SmF. The kinetics on submerged fermentative production of keratinase is also discussed.

Chapter 3 – “Materials and Methods” describe about the steps involved in substrate preparation, various microorganisms used and their maintenance and the analytical procedure for determination of keratinase activity and cell mass determination. The experimental procedure followed is also given.

Chapter 4 – “Results and Discussion” summarizes overall results of the keratinase enzyme production by submerged fermentation. This includes (I) Classical method of optimization of keratinase, (II) Screening of nutrients for maximum keratinase production by Plackett-Burmann design, (III) Optimization of selected ingredients using CCD and RSM for the high yielding strains and (IV) Process parameter optimization for major variables such as incubation period, temperature, initial pH, substrate concentration and inoculum size for maximum keratinase production.

Chapter 5 – “Kinetics and Modeling” The kinetics of keratinase enzyme production was studied under optimum conditions using the optimized media using B.subtilis and S.griseus with CFP as substrates. Various models were tried for growth kinetics, product formation kinetics and substrate utilization kinetics will be presented.

Chapter 6 – “Conclusions” Summarizes the production results obtained in the present research work and suggestions for future studies of keratinase