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SCREENING, CHARACTERIZATION AND PURIFICATION OF KERATINASE PRODUCED BY *Bacillus licheniformis* ISOLATED FROM NAMAKKAL POULTRY FARM SOIL.

PROJECT REPORT

Submitted by

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Register No: 0820203017

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Of

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in

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PROJECT WORK -PHASE II

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This is to certify that the project entitled **SCREENING, CHARACTERIZATION AND PURIFICATION OF KERATINASE PRODUCED BY *Bacillus licheniformis* ISOLATED FROM NAMAKKAL POULTRY FARM SOIL** is the bonafide record of project work done by **C.VIGNESHWARAN Register No: 0820203017** of M.Tech during the year 2009-2010.

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I affirm that the project work titled **Screening, Characterization and Purification of Keratinase Produced by *Bacillus licheniformis* isolated from namakkal poultry farm soil** being submitted in partial fulfilment for the award of **M.Tech (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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BSA	Bovine Serum Albumin
SDS- PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electrophoresis
kDa	Kilo Dalton
DEAE	Diethyl Amino Ethyl
TCA	Trichloro acetic acid
rpm	Rotation Per Minute

Abstract

ABSTRACT

Keratin are insoluble fibrous proteins found in hair, wool, feather, nail, horns and other epithelial covering which is rich in beta helical coil linked through cysteine bridges. Keratinase (EC 3.4.4.25) belongs to class hydrolase which are able to hydrolyse insoluble keratins more efficient than other proteases. The bacteria *Bacillus licheniformis* showing higher keratinase activity was screened out of the ten different bacterial strains isolated. The ability of *Bacillus licheniformis* to utilize chicken feather powder as a substrate was tested. The keratinase was purified to homogeneity by ion exchange chromatography. The purified enzyme exhibited a high specific activity (543 U mg^{-1}). The enzyme was monomeric and had a molecular mass of 37kDa Similarly optimum temperature and pH for the enzyme activity was found to be 60°C and 7.0 respectively. The k_m and V_{max} values were 0.22 mg/ml and 0.01 U/ml respectively. The enzyme is stable (30-40°C) and active around wide pH range (6-8). Among the various metal ions zinc, magnesium were found to enhance the enzyme activity where as mercury, copper, cadmium, 1, 10 phenanthroline and EDTA completely inhibit the enzyme activity. It was found from this study, organism such as *Bacillus licheniformis* isolated from poultry soil is a potential candidate for degradation of feather and for dehairing process in leather industry.

Introduction

1. INTRODUCTION

Keratins are a major class of structural proteins that are highly resistant to biological degradation. Common enzymes which break down protein, such as trypsin, do not affect keratin. Keratins, like other proteins, are made of a long string of various amino acids, which fold into a final 3-dimensional form. Alpha helix and beta sheet are common parts of such a 3-dimensional form. Long, thin biological structures are often contains alpha helix, while flat structures are often built from beta sheets. Some keratins are rich in alpha helix structures while others are mostly beta sheet.

Keratins are divided into two categories, the soft keratin and hard keratin. The outer layer of epidermis of the skins is an example of soft keratins. Hair, wool and feather though classified as hard keratins also have some histological characteristics common with soft keratin. Horns, hoofs and nails are classified under the hard keratins.

Chemically the difference between the soft and hard keratins is known by the percentage of sulphur content which is as high as 5% in the case of hard keratin and as low as 1% in soft keratins. Wool and feather keratins constitute about 3% of sulphur content. The lipid is higher in the soft keratins and lower in hard keratins. Hence the thermal stability of the keratins is low and those hard keratins are higher.

Keratin is insoluble in water, aqueous solution of neutral salts and organic solvents. The postulated basic polypeptide subunit of keratin has a molecular weight of approximately 10,400 units. The disulfide bonds of keratin stabilize the stereo chemical configurations that are responsible for the resistance of these substances to chemical and enzymatic attack

The general process takes place in the production of keratin in animal feathers, hairs, hoofs, nails and horn etc. Keratin contains more than twenty two amino acids especially in the helical regions of their structure. Of the amino acids present in the keratin, important amino acids are Cysteine, Arginine, Serine and Glycine. In the amino acids analysis the presence of high portion of cysteine disulphide linkages are noticed. Nearly all the known amino acids are present in significant amounts although there is very little histidine, tryptophan, and methionine.

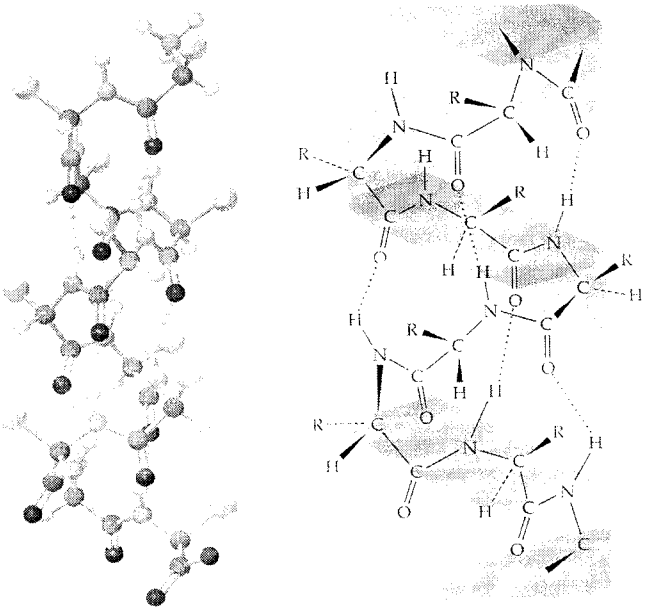


Figure – 1: 3D Structure of Keratin

Diaminoacid cysteine makes an important contribution to the stability of protein, as it contains two α-carbonyl groups and can therefore form part of two adjacent polypeptide chains linking them together through the disulphide group of cysteine residue.

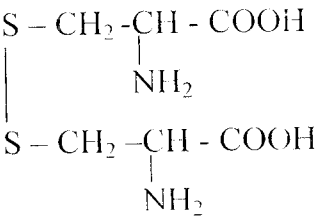


Figure - 2: Structure of Cysteine

Types of keratin:

Alpha keratin - It is present in wool, hair and horn. It is in the form of folder chain.

Beta keratin - It is present in feather in the form of peptide chain.

Keratin are formed in the outermost living cells of the skin, they are structure protein of hair, wool, feathers, horn, nail and hoofs. The cysteine seems to be fundamentally involved in the insolubility of keratin and resistance to enzymes.

Keratinase (EC.3.4.4.25) belong to the group of serine hydrolyses that are capable of degrading keratin. Keratinase have broad substrate specificity and are active against both

common proteases (Goddard and Michaelis, 1934; Papadopoulos, 1986). The feather can be hydrolyzed by keratinase which is a proteolytic enzyme specific to keratins (Friedrich *et al.*, 1999). The hydrolysis step is the key step in converting a polymer structure such as feather protein (keratin) into a useful and nutritious feed ingredient. Hydrolysis is the introduction of water molecules into a structure and can be affected by steam under pressure, chemical or enzyme action or a combination of all three. In the case of feather hydrolysis, some of the polymer (10-50 million Dalton molecular weight) is broken down into shorter chains, above and below 10,000 Dalton molecular weight.Feathers are produced in large amounts as a waste by product of poultry processing plant.

A current value added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments. These methods can destroy certain amino acids and decrease protein quality and digestibility (Riffel and Brandelli, 2006).An enzyme which degrades keratin is a keratinase, while a beta-keratinase is an enzyme capable of degrading beta-keratin. An enzyme which degrades keratin can also be described as having keratinolytic activity. This occurs commonly in nature in some microorganisms and, in some cases, preferably grow on keratinaceous substrates has supported the general belief that certain microorganisms can digest keratin (Noval and Nickerson, 1959).

Keratinase producing bacteria are *Bacillus sp.* (Zerdani *et al.*, 2004). *Bacillus licheniformis* (Ramnani *et al.*, 2005; Korkmaz *et al.*, 2004; Manczinger *et al.*, 2003; Williams *et al.*, 1990). *Burkholderia, Chryseobacterium, Pseudomonas, Microbacterium sp* (Brandell and Riffel, 2006) were isolated and was studied with respect to different parameter. This enzyme has been produced by fungi, including the species of *Aspergillus, Onygena, Absidia* and *Rhizomucor* (Friedrich *et al.*, 1999), some species of dermatophytes, including *Trichophyton mentagrophytes, T. rubrum, T.gallinae, Microsporum canis* and *M. gypseum* (Wawrzkiewicz *et al.*, 1991).

The enzyme is a potential enzyme for removing hair and feather in the poultry industry (Takami *et al.*, 1992), for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry (Williams *et al.*, 1991), and for clearing obstructions in the sewage system during waste water treatment and eco-friendly de-hairing process in leather industry. However, much current research is centered on the potential use of keratinase of

modification have been used for various industrial applications. In India sizable quantity of waste comprising of keratin proteins is available, particularly in place where animal hides and skins are processed and poultry dressing plants are located. In spite of many options available, no profitable solution is forthcoming to converts this waste into suitable products. Also the disposal involves high costs thereby neglecting suitable disposal to protect our environment. Keratinolytic enzymes have been studied from a variety of fungi, but to a lesser extent in bacteria. With this background, this study was designed with to select and identify feather degrading bacteria from Namakkal poultry farm soil and to study the characterization and purification of keratinase producing bacteria.

Objective of the Study

2. OBJECTIVES

- To screen for keratinase producing bacterial species (*Bacillus sp.,*)
- To characterize and evaluate the keratinase using different parameters like pH, temperature, substrate concentration and inhibitors.
- To purify keratinase enzyme using ammonium sulphate fractionation and ion exchange chromatography.
- To study the application of keratinase in industries

3. REVIEW OF LITERATURE

3.1 Keratin

The crystal kinetics of keratin was determined and postulated that keratin consists of ordered helical micro fibrils embedded in a non-crystalline matrix, which has longitudinal zones with differing characteristics (Skertchly, 1962). Many features of X-ray diffraction diagram of keratin could be explained in terms of linear aggregates of small beta crystallites (Fraser and Macrae, 1962). Also the feather keratin contains fibrillar elements (microfibrils) about 30A° in diameter.

The chicken body feather could be treated with a solution containing mercaptoethanol and a protein denaturants such as urea, or with a solution containing mercaptoethanol and an anionic surface active agent such as sodium dodecyl benzene sulphonate (SDBS) or sodium laurylsulphate (SLS) (Toyoda *et al.*, 1967). Current research is centered on the potential use of keratins of bacterial origin for the industrial treatment of keratin-containing compounds (Allpress and Mountain *et al.*, 2002). The solubilization of human hair with 2-mercaptoethanol solutions containing various protein denaturants, i.e., urea, guanidine, ethyl carbonate, sodium salicylate and ammonium thiocyanate. In addition, the amino acid compositions of solubilized products and insoluble residues were compared and investigated.

Keratin comprises long polypeptides chains, which are resistant to the activity of non-substrate-specific proteases. Adjacent chains are linked by disulphide bonds thought responsible for the stability and resistance to degradation of keratin (safranel and goose *et al.*, 1982).

The solubility of cattle and pig hair keratins. They collected the samples from steer hides and pig skins with razors and treated with sodium thioglycolate or formic acid. The effects of concentration of the reducing or oxidizing agent, incubation temperature and the time on the solubility of hair keratins were examined. (Kohkichi *et al.*, 1993)

3.2 Keratinase

Cross linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic enzyme; however, reduction of cystine bridges may have a significant influence on keratin degradation (Noval *et al.*, 1958). Purified keratinase was covalently immobilized on succinamidopropyl glass by the sequential activation –

material with an enzyme material isolated from a culture of *Micrococcus serenities* at the optimum pH and temperature for activity was about 8.2 and 40°C.

Bacillus species FK 28 was inoculated into the medium containing different concentration of feather (1 – 5%). Keratinase is produced maximally in the medium containing 1% feather meal. The keratinase is produced in the range of 0.7 – 2.6 U/ml at 37°C with an initial pH of 7.5 at 150 rpm (Dakrong Pissuwan and Suntorsuk 2001). Isolation, Identification and Characterization of feather degrading bacterium namely *Bacillus species* from poultry waste. The isolate is a rod shaped bacterium which appeared singly and in chains and the strain has an activity of 50 U/ml (Savitha Joshi *et al.*, 2007).

3.3 Physiology of keratinase production

Microbial keratinase are predominantly extra cellular when grown on keratinous substrate; however, a few cell-bound (Friedrich and Antranikian 1996; Onifade *et al.*, 1998) and intracellular keratinases have also been reported (El-Naghy *et al.*, 1998; Onifade *et al.*, 1998).

Keratinases are largely produced in a basal medium with a keratinous substrate. Most of the organisms are capable of using keratin as the sole source of carbon and nitrogen (Williams *et al.*, 1990; El-Naghy *et al.*, 1998).

Most reports available on keratinases group them as inducible enzyme; however, few constitutive keratinases have also been reported (Gassesse *et al.*, 2003).

3.4 Sources of keratinase enzyme

The productions of keratinase enzyme from different organisms are shown in (Table1) below

TABLE 1. Sources of keratinase enzyme

Microorganism	Reference
Bacillus licheniformis	Ramnani *et al.*, 2005
Pseudomonas sp.	Brandell and Riffel, 2006
Streptomyces pactum	Bockle *et al.*, 1995
Rhizomucor	Friedrich *et al.*, 1999
Trichophyton mentagrophytes	Wawrziewicz *et al.*, 1991
Fervidobacterium pennavorans	Friedrich and Antranikian, 1996
Thermoactinomyces candidus	Ignatova *et al.*, 1999

3.5 Degradation of feathers using bacteria

A feather degrading cultures was enriched with isolates from a poultry wastes digester and adapted to grow with feathers as its primary source of carbon and sulfur as its energy. The microorganisms were identified as *Bacillus lichiniformis* PWD-1. The maximum growth (approximately 10^9 cells per ml) at 50°C and at pH 7.5 occurred 5 days post inoculated on 1% feathers substrate to 2 parts *Bacillus lichiniformis* PWD-1 culture (10^7 cells per ml) feather hydrolysis was evidence as free amino acids produced in the medium. These data indicate a potential biotechnique for degradation and utilization of feather keratin (Williams, *et al.*, 1990).

From a hot spring of the Azores islands a *Thermophilic bacterium* was isolated this strain was fist known extreme thermophilic that is able to degrade native feathers at high temperatures. The enzyme system converts feather meal to amino acid and peptides. The

bacteria were tested for their abilities to grow on feather and produce stable keratinolytic enzymes. None of the tested extremophlic microorganisms was able to attack this substrate in a native form (Andrea Friedrich and Antranikian, 1996).

Production of Extra Cellular feather degrading enzyme by *Bacillus lincheniformis* isolated from poultry farm. Rod shaped bacteria along with coccus has been found. But when it is streaked in feather meal agar plate the rod shaped bacteria produced clear zones. This has been identified and characterized and it is found to contain an optimum pH and temperature of 8 and 35°C. The optimum conditions for keratinase synthesized by *Bacillus lincheniformis* which is determined is an essential step for the production of adequate amount of enzyme (Tamilmani *et al.*, 2008). Isolation and identification of a thermotolerant feather degrading bacterial strain *Bacillus licheniformis* from that soil as well as purification and properties of its keratinase were investigated. The optimum pH and temperature for the enzyme were 8.5 and60°C, respectively was reported (Suntorsuk *et al.*, 2001).

The degradation of poultry waste by using the strains *Bacillus licheniformis* and *Sterptomyces species* these two bacterial inoculums were found to degrade keratin more quickly and more completely.

Feather degrading bacteria were isolated from poultry waste. Among those isolates, three strains identified as *Bacillus subtilis, Bacillus pumilis & Bacillus cereus* degraded feather effectively and produced 142, 96 &109 Units of keratinolytic activities respectively

3.6 Pretreatments methods for hydrolysis of poultry feathers

Because of the complex, rigid and fibrous structure of keratin, poultry feather is a challenge to anaerobic digestion. It's poorly degradable under anaerobic conditions. However, application of appropriate pretreatments methods hydrolyzes feather and breaks down its tough structure to corresponding amino acids and small peptides For more than half a century many studies have been performed and various pre treatment methods have been applied to improve the digestibility of feather meal as well as development of its nutritional value for production of a dietary protein feedstuff for animals (Wang, X *et al.*, 1997) . These pretreatments methods may also enhance feather for keratinase potential. Feather meal treatment methods are usually categorized into two groups: hydrothermal treatments and microbial keratinolysis (Onifade *et al.*, 1998)

3.7 Biological pretreatment of chicken feathers

Biodegradation of feathers is another alternative method. Some bacterial strains can produce keratinase proteases which have keratinolytic activity and are capable to keratinolyse feather β-keratin. These enzymes help the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of β-keratin Savitha G. Joshi 2007

Various keratinases from different microorganisms such as *Bacillus sp.* (Williams *et al.*,1990). *Bacilluslicheniformis*, *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Microbacterium sp.* (Brandelli *et al.*, 2006). has been isolated

Microbial proteases are classified into acidic, neutral, or alkaline groups. depends on the required conditions for their activity and on the characteristics of the active site group of the enzyme, i.e. metallo-, aspartic- ,cysteine- or sulphydryl- or serine-type. Alkaline proteases which are active in a neutral to alkaline pH, especially serine-types, are the most important group of enzymes used in protein hydrolysis, waste treatment and many other industrial applications. Alkaline protease from *Bacillus subtilis* was used for the keratinolysis of waste feathers (Mandal *et al.*, 2009) Subtilisins are extracellular alkaline serine proteases, which catalyse the hydrolysis of proteins and peptide amides. Savinase is one of these enzymes; Alcalase, Esperase and Maxatase are others. These enzymes are all produced using species of Bacillus. Maxatase and Alcalase come from *Bacillus licheniformis* Esperase from an alkalophilic strain of a *Bacillus licheniformis*, and Savinase from an alkalophilic strain of *B. amyloliquefaciens*. (Mandal *et al.*, 2009)

An important advantage of enzyme treatment method is fully biodegradability of enzymes by themselves as proteins. Hence, unlike other remediation methods, there is no build up of unrecovered enzymes or chemicals that must be removed from the system at the end of degradation process. (Mandal *et al.*, 2009)

3.8 Microbial hydrolysis of keratin

The degradation of native keratins by *Streptomyces fradiae*. They also characterized the amino acid using chromatographic methods

A keratinolytic protease from the fungus *Doratomyces microsporus* was investigated for its ability to hydrolyze different native proteins. The purified enzyme was incubated for

decreased in the following order: skin keratin > nail keratin >hair keratin. Feather keratin and collagen could not be hydrolyzed (Friedrich *et al.*, 1996).

With the aim of efficiently utilizing livestock waste as a protein resource, we studied their enzymatic hydrolysis. Particles of a mixture of horn and hoof from cow and buffalo were used. For enzymatic hydrolysis of the substrate, heat treatment was necessary prior to digestion and the conditions for the enzymatic reaction were determined to be as follows: reaction time 30 to 60 minutes; pH 8.3; temperature, 50°C; weight ratio of substrate to enzyme, 1:0.05; and concentration of substrate.62.5g/l (Kenji Kida *et al.*, 1995).

The cross linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic enzyme; however, reduction of cystine bridges may have a significant influence on keratin degradation (Noval *et al.*, 1958)

The *Streptomyces species* 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. 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 valorization of keratin containing wastes, or the depilation process in the leather industry (Letourneau, et al., 1998). Keratinase 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 5mM mercaptoethanol. 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 $(OH)_2$. Ekta Tiwary *et al.*, 2010)

3.9 Molecular weight

Keratinase from the *Bacillus subtilis* strain have a molecular weight range between 64 and 69 kDa (Balaji *et al.*, 2008). Molecular weight of keratinases ranges from 18 to 200 kDa. The lowest value 18kDa for S.albidoflavus *SK1*- 02 (chitte *et al.*, 1999), the highest of 200 kDa for *kocuria rosea* & *F. islandicum*. The keratinolytic serine protease from *Streptomyces pactum* DSM 40530. The enzyme was purified by casein agarose affinity chromatography and had a molecular weight of 30,000 Daltons (Bockle *et al.*, 1995).

A stable aqueous solution of reduced keratin was prepared by extracting the protein

degrading activity was observed at 30-37°C and the keratinolytic enzyme had a temperature optimum of 45-65°C. (Patricia and Adriano 2005). The Optimum keratinolytic activity was observed at 40°C. The enzyme also showed to be stable between 40 and 60°C.

3.10.3 Effect of Substrate concentration

Keratinases have broad substrate specificity and are active against both soluble & insoluble proteinaceous substrates. Among soluble protein, they possess the ability to hydrolyze casein, gelatin, BSA & haemoglobin, whereas among insoluble proteins, they hydrolyze feathers, wool, silk, collagen, elastin, horn, stratum corneum, hair, azokeratin and nail.

The PWD-1 keratinase is an enzyme that was originally purified from the growth medium of *Bacillus licheniformis* PWD-1 (Williams et al., 1990; Lin et al., 1992). PWD-1 keratinase hydrolyzes a broad range of protein substrates including casein, collagen, elastin and keratin, and displays higher proteolytic activity than all other proteases tested (Shih, 2001) used PWD-1 keratinase to produce hydrolyzed feather meal by incubating commercial feather meal with cell-free keratinase over night. The hydrolyzed feather meal was demonstrated to replace soybean meal at 7% dietary level. However, supplementing poultry diets with PWD-1 keratinase directly as a feed additive has not been previously investigated

3.10.4 Effect of Enzyme Inhibitors and Metal Ions

Keratinase from the *Bacillus subtilis* strain was completely inhibited by ethlyenediamine tetraacetic acid (EDTA) and 1, 10 phenanthroline, and remain unaffected by phenylmethanesulfonyl fluoride (PMSF) whereas iodoacetamide inhibited considerably zinc, magnesium, calcium manganese and nickel enhances the enzyme activity (Balaji *et al.*, 2008). The keratinolytic activity was inhibited by PMSF and partially inhibited by EDTA and iodoacetamide (Woraput suntornsuk *et al.*, 2005). Reducing agents like dithiothreitol (DTT), B-mercaptoethanol, reduced Glutathione, Cysteine and Sodium sulfite enhance keratinolytic activity, indicating that these enzymes are Thiol-activated (chitte *et al.*, 1999).

Keratinases mostly belong to the class of serine or metalloproteases irrespective of the micro organism. Therefore, phenylmethanesulfonyl fluoride (PMSF) Ethlene Diamine- Tetra Aceticacid & 1, 10- O-Phenanthroline (Dozie et al., 1994; Riffel *et al.*, 1999). Reducing

The keratinolytic activity was not inhibited by EDTA, DMSO and Tween 80. On the other hand, CaCl2, ZnCl2, and BaCl2 slightly inhibited the keratinolytic activity. (Daniel M. T. Tapia et al., 2008).

3.11 Purification of keratinase

Keratinase from *Bacillus licheniformis* was purified to homogeneity by three step chromatography. The purified enzyme exhibited a high specific activity (218 U mg^{-1}) with 86-fold purification and 25% yield. (Woraput suntornsuk *et al.*, 2005).

The keratinases from recombinant *P. pastoris* (pPZK3) and *B. megaterium* MS941 (pWAK3) were purified to 67.7- and 85.1-folds, respectively, through affinity chromatography. The purified keratinases had the specific activity of 365.7 and 1277.7 U/mg, respectively (Selvaraj Radha *et al.*, 2009)

Keratinase from *Bacillus subtilis* was purified (45-fold) by using ion exchange and gel filtration chromatography.(Balaji et al., 2008)

Crude keratinase from *Bacillus licheniformis* was purified by salt precipitation, dialysis and column chromatography and maximum activity was found. (Tamil Mani *et al.*, 2008)

A keratinase was isolated from the culture medium of feather-degrading *Bacillus licheniformis* PWD-1 by use of an assay of the hydrolysis of azokeratin. Membrane ultrafiltration and carboxymethyl cellulose ion-exchange and Sephadex G-75 gel chromatographies were used to purify the enzyme. The specific activity of the purified keratinase relative to that in the original medium was approximately 70-fold. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis analysis and Sephadex G-75 chromatography indicated that the purified keratinase is monomeric was found. (Xiang Lin *et al.*, 1992)

Keratinase from *Trichophyton mentagrophytes* and it was partially purified with sephadix G-100 gel filtration and some biochemical characteristics of the purified enzyme were determined

The keratinase from *Doratomyces microspores* was purified to homogeneity using hydrophobic interaction chromatography followed by gel chromatography maximum activity was obtained.

The keratinase from *Streptomyces species*A_{11} and it was found to degrade keratin from human epidermis. They purified the enzyme by DEAE-cellulose column.

The keratinolytic serine protease from *Streptomyces pactum DSM 40530*. The enzyme was purified by casein agarose affinity chromatography and had a molecular weight of 30,000 Daltons (Bockle *et al.*, 1995)

3.12 Application of keratinase enzyme

Keratin, as well other insoluble proteins, is generally not recognized as a substrate for common proteases. Its hydrolysis is, however, affected by specific proteases (Keratinases) which have been found in some species of *Bacillus* (Williams *et al.*, 1990). These enzymes may have a use in the biotechnological application of keratin-containing wastes, as well as in the leather industry where they may have potential in the elaboration of non-polluting processes (Mukhopadhya and Chandra, 1993).

Current research is centered on the potential use of keratins of bacterial origin for the industrial treatment of keratin-containing compounds. (Allpress, Mountain *et al.*, 2002).

In view to protect the environment an attempt has been made in this to convert the keratinous waste into a suitable valuable product. The keratinous waste chosen for the present study are chicken feather material. This material is available in our country presently and largely wasted. These wastes are converted into keratin hydrolysate (KH) by controlled enzyme hydrolysis (bacterial degradation) to get a soluble product used in leather processing.

Keratin, a fibrous protein forming main structural constituents of feather, hair, wool, horn, hoof. It is abundantly available as a by-product from poultry, slaughterhouse, tanning and fur processing industry. Keratins though find application in food, pharmaceutical, cosmetics and fertilizer industry, considerable amount of product is wasted repeatedly. Keratins are difficult to degradation and their disposal leads to environmental problem. Research is being done globally to utilize the waste. Keratin hydrolysates find potential application in leather tanning industry

3.13 Predicting the structure

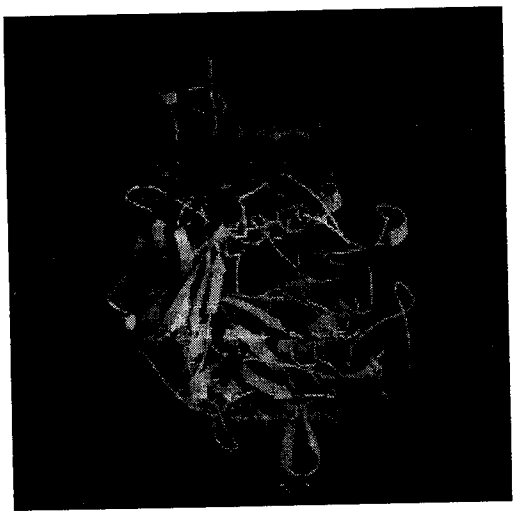


Figure 3: crystal structure of keratinase

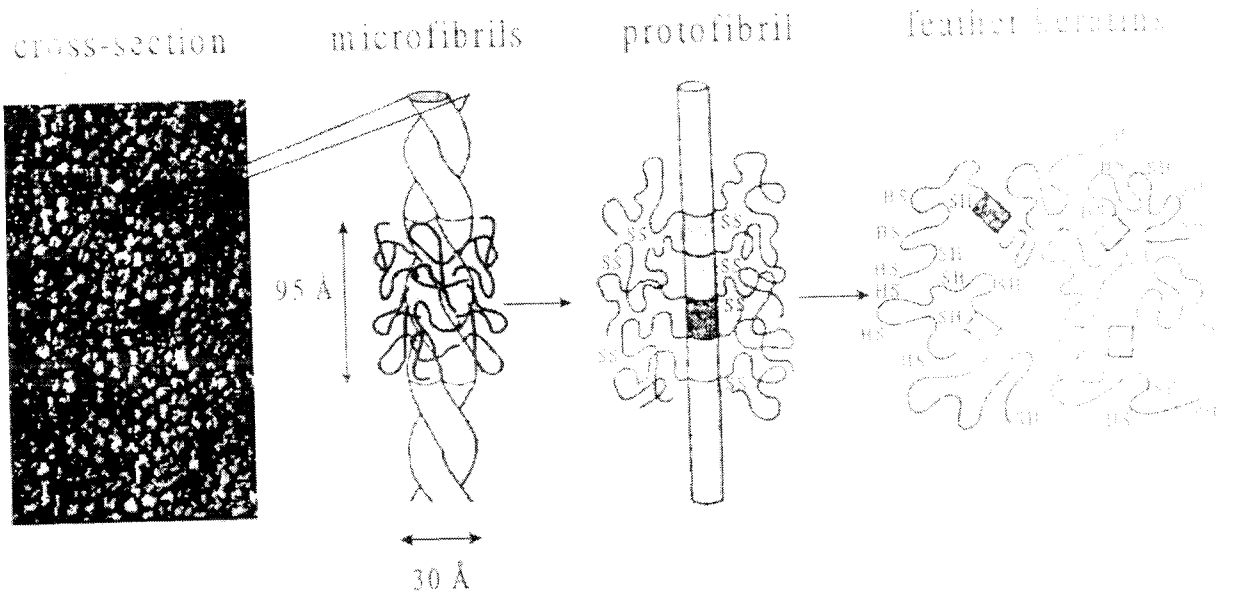


Figure 3. Scanning electron micrograph of a cross-section of the feather calamus and a molecular model for the microfibril of feather keratins made up of two twisted protofibrils. These protofibrils are formed by small β–sheet crystallites. The β–sheet is probably formed by the central portion of a feather keratin molecule, the N- and C-terminal of the molecule form the amorphous matrix, stabilized by cystine, in which the fibrils are embedded. Six cysteine residues are present in the N-terminal region, which only

Materials and Methods

4. MATERIALS AND METHODS

4.1 Isolation of microorganism

Samples (soil and feather) were taken from the poultry farm in the town of Namakkal (Tamilnadu). Serial dilution for each sample was prepared by adding 1 g of the soil sample to 9 ml of sterile saline. Then serial dilution up to 10^{-9} was done using sterile saline. All the dilutions were plated on Nutrient Agar medium and incubated at 37°C for 24 hours. The colonies appeared was checked for the presence of spore and streaked onto agar slants for further characterization.

4.2 Characterization

All the collected strains were grown on Nutrient Agar medium for fresh cultures. Spore production and localization were examined by microscopic observations. The identification was done according to the method described by Larpent and Larpent-Gourgaud (1985).

4.3 Identification of Isolated feather degrading bacteria

The organism was identified and confirmed by carrying out tests like Gram Staining, Spore staining, Motility test, Catalase Test, Starch hydrolysis test and casein hydrolysis test

4.4 Screening on the feather medium

The strain that streaked on the agar plate containing Salt, Vitamin, Yeast extract and keratin (Feather powder). The plates are incubated between 35°C and 60°C. The bacterial colonies on the agar plate which shows growth are retained

4.5 Screening for Keratinolytic bacteria

Among the different bacterial colonies obtained on the spread plated agar plate. Ten different morphologically different bacterial colonies were identified and each inoculated onto a sterile feather meal agar plate. The inoculated plate was then incubated at 37°C for 48 hours. The strain that shows high zone of clearance was selected and it was sub cultured. The strain was further grown on nutrient broth containing feather meal and kept for incubation at 40°C for 7days with shaking at 150 rpm. Then the culture supernatant was assayed for kerotinolytic activity. The strain, which degraded keratin effectively and it was further identified by phenotypic study.

4.6 Protein quantification

Protein concentration was measured by the method of Lowry using Bovine serum albumin (BSA) as standard. Protein assay mixture consisted of 0.05ml of sample and standard of 0.05mg of BSA prepared were taken as working standards of different volumes, 0.15ml of distilled water and 0.1ml of 1N Folin's reagent were added. Then the contents were incubated at room temperature for 30 minutes. A reaction mixture containing distilled water was used as blank. Blue colour developed in the standard and tests were measured at 660nm

4.7 Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of (Wawrzkiewicz et *al*., 1987). Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a hot air oven at 100 °C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −70 °C for 2 h, followed by centrifugation at 10, 000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40 °C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20 ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1M Hydrochloric acid and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer (pH 7.0).

4.8 Keratinase activity assay

The keratinolytic activity was assayed 1.0 ml of crude enzyme properly diluted in Phosphate buffer (0.05 M of pH 7.0) was incubated with 1 ml keratin solution at 50 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4M Trichloroacetic acid (TCA). After centrifugation at 1450×g for 30 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution.

One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A_{280}) (Gradisar *et al*., 2005) with the control for 0.01 per minute under the conditions described above.

4.9 Feather degradation by weight loss method

Feather was first washed with detergent followed by several washing with water. It was dried at 60 °C and stored at room temperature till use. To carry out percent feather degradation, 250 mg native feather was added in 50 ml, 25 mM, phosphate buffer (pH 7) and autoclaved at 15 psi for 15 min. Keratinase (concentrated by ultra filtration) was filter sterilized by 0.2 µm and keratinase was used for cell free degradation. Feather degradation was carried out at 50°C, for 24 h or for the specified time period at 150 rpm. Reaction mixture was filtered through preweighed Whatman filter paper No. 1 and dried at 80°C for 4 h (or till a constant weight was achieved) to determine residual feather. Control was also set for 24 h with inactivated enzyme. The time of feather degradation was studied by determining percent feather degradation after 2, 4, 6 and 8 h.

4.10 Characterization of crude enzyme

4.10.1 Effect of pH

The optimum pH of the crude enzyme was found by dissolving the keratin at various buffers using 0.05 mol/L acetate buffer (pH 4-5.5), 0.05 mol/L phosphate buffer (pH 6-7.5), 0.05 mol/L Tris-Hcl (pH 8-9), Based on the above mentioned pH the keratin solution is prepared for various buffers. The experiment on the effect of pH on enzyme stability was carried out by incubating the enzyme solution at pH ranges of 4-9. Then the enzyme activity was determined by the standard enzyme assay.

4.10.2 Effect of temperature

With the optimum pH of the crude enzyme as constant the optimum temperature was found by incubating the enzyme with the substrate (keratin solution prepared according to optimum pH) at varying temperature range from 30 to 80°C. The experiment on the effect of temperature on enzyme stability was carried out by incubating the enzyme solution at temperature ranges of 30-80°C Then the enzyme activity was determined by the standard enzyme assay.

4.10.3 Substrate concentration

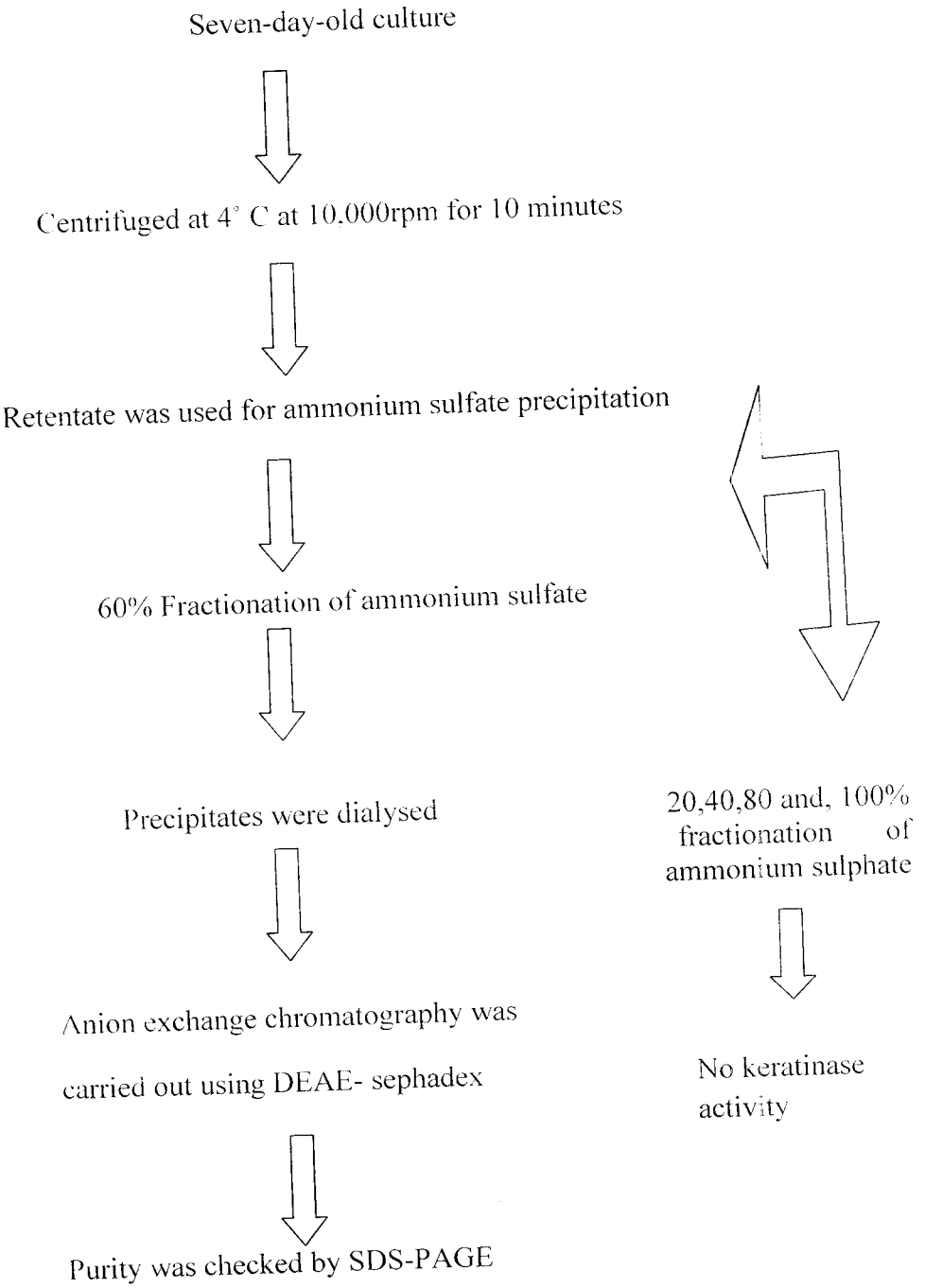
The k_m and Vmax value for the crude keratinase is determined by using different concentrations of keratin. For this stock solution of keratin is prepared by dissolving 0.5 g in 50 ml phosphate buffer of pH 7. The stock solution is diluted for different concentrations (0.1-0.7 g) in a series of test tubes using phosphate buffer.

4.10.4 Effect of Enzyme inhibitors and Metal ions

To study the influence of enzyme inhibitor, crude keratinase (1ml) was incubated with varying concentration (1-3mM) of 1.10 Phenanthroline and EDTA in 0.05mol/l phosphate buffer (pH 7) at 37°C for 30min. The effect of divalent cation on keratinolytic activity was determined by incubating the crude enzyme in the presence of Zinc, magnesium, copper, mercury, cadmium (Zn^{2+}, Mg^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}) at (1-3mM) concentration for 1h at 37°C. Then the enzyme activity was determined using lysis of feather powder as described above

4.11 Purification of enzyme

With the literature obtained the enzyme was purified with the following steps



4.11 Purification

The isolated strain was further grown on nutrient broth containing feather meal and kept for incubation at 40°C for 7days with shaking at 150 rpm. Then the culture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The culture supernatant was used for ammonium sulphate precipitation.

4.11.1. Ammonium sulphate precipitation

The crude enzyme was purified from the culture supernatant fluid using ammonium sulphate. For the purpose, 20,40,60,80 and 100% of ammonium sulphate saturation were used for the precipitation of enzymes. The respective levels were mixed to a volume of crude enzyme filtrate and kept at 4°C for one to two hours with continuous stirring. Then the mixture was kept overnight. The precipitates were collected by centrifugation at 10,000 rpm for 15 minutes. The resultant pellets were resuspended in phosphate buffer (50mM, pH 7.0) and dialyzed at 4°C.

4.11.2. Dialysis

After precipitation, the ammonium sulphate present in the enzyme solution was removed by subjecting the solution to dialysis in phosphate buffer (50mM, pH 9.0) at 4°C with intermittent change in buffer for every 12 hours. The content of dialysis bag was centrifuged to remove precipitates, if any. The dialyzed sample was then collected and analyzed for kertinase activity. The optimum activity at a specific concentration of ammonium sulphate reflects the best concentration to attain maximum enzyme recovery.

4.11.3. Ion exchange chromatography

4.11.3.1. Regeneration of the DEAE-Sephadex column

DEAE-Sephadex column (2.3 x 12.2cm) was regenerated by washing with 250ml of Tris-HCl buffer (0.1M, pH 8.3) containing 0.5M NaCl followed by passing 300ml of sodium acetate buffer (0.1M, pH 4.5) containing 0.5 NaCl. The regenerated DEAE-Sephadex was equilibrated with 400ml of phosphate (25mM, pH9.0). DEAE-Sephadex was kept at 4°C.

4.11.3.2. DEAE-Sepharose chromatography

The enzyme was loaded on to a DEAE-Sephadex column (2.3 x 12.2 cm)

linear salt gradient 0.5M NaC1 in equilibration buffer (25mM, PH 7.0). Fractions were collected at a flow rate of 30ml/hr using collector. All the fractions were checked for protein (A280) and keratinase activity (A540). The fractions having maximum activity were pooled, dialyzed and lyophilized.

4.11.4 SDS –PAGE

The sodium dodecyl sulphate -polyacrylamide gel electrophoresis was used to check its purity. The lammeli method was followed and BSA, Casein was used as Standard protein marker. The stacking and separating gel mixture of 4% and 10% was used respectively. The sample was prepared with sample solubilizing buffer. The graph was plotted with the Rf values and molecular weight of the protein. With the relative mobility of the protein the molecular weight was calculated. The sample was kept in boiling water bath for five minutes prior to load and it was mixed with dye Coomassie brilliant blue. The purified sample was analyzed and compared with the protein marker to find its size

5. RESULTS

5.1Bacterial selection and identification

A total of ten keratinase producing bacteria were screened for feather degrading properties. It was found that the NS-3 strain was the only feather degrading strain (shown in Table-2) capable of growing and degrading feather at 40°C within 7 days.

TABLE-1: Keratinase production by bacterial isolates from soils in Namakkal poultry farm.

Isolate	Keratinase (U/ml)
NS-1	0.21
NS-2	2.36
NS-3	10.76
NS-4	0.17
NS-5	4.94
NS-6	0.98
NS-7	2.00
NS-8	2.38
NS-9	3.35
NS-10	1.56

Abbreviations: NS- (Namakkal soil strain)

The NS-3 strain (NS- Namakkal soil) which appeared singly or in chain and was straight rods, Gram positive and endospore-forming organism. It was aerobic, motile, and strong oxidase- and catalase positive. Additional morphological, physiological and biochemical test were conducted as shown in (Table 3). The feature agreed with description of Bergy's Manual of systematic Bacteriology. It was identified to be *Bacillus licheniformis*. On the basis of morphological characteristics and phenotypic studies, the strain was found to be *Bacillus* sp.having 99% similarity with *Bacillus licheniformis*. From this result, the NS-3 strain was identified as *Bacillus licheniformis*.

TABLE-3: Results Of Morphological and Biochemical Tests for *Bacillus licheniformis*

EXPRIMENTAL DETAILS	OBSERVATIONS
Gram stain	Positive
Shape and arrangement	Rods in Single and in Chains
Endospore stain	Positive
Litmus milk reactions	Peptonization
Carbohydrate fermentations with lactose	Acid with Gas
Sucrose	Acid with Gas
Dextrose	Acid with Gas
Nitrate reduction	Positive
Motility test	Positive
Indole production	Positive
Methyl red test	Negative
Citrate utilization	Positive
Catalase activity	Positive
Starch hydrolysis	Positive
Caesin hydrolysis	Positive
Urease activity	Positive

Figure-4: Starch Hydrolysis Test

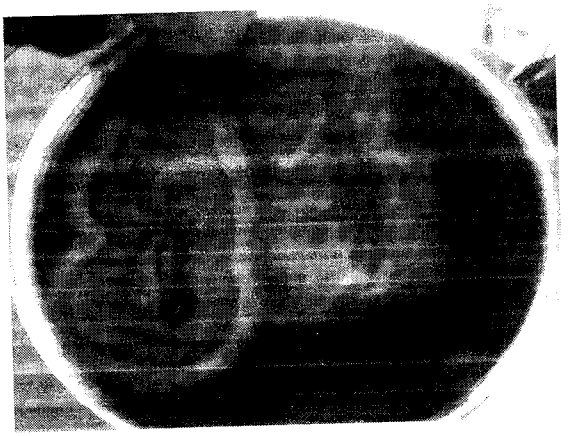


Figure-5: Casein Hydrolysis Test

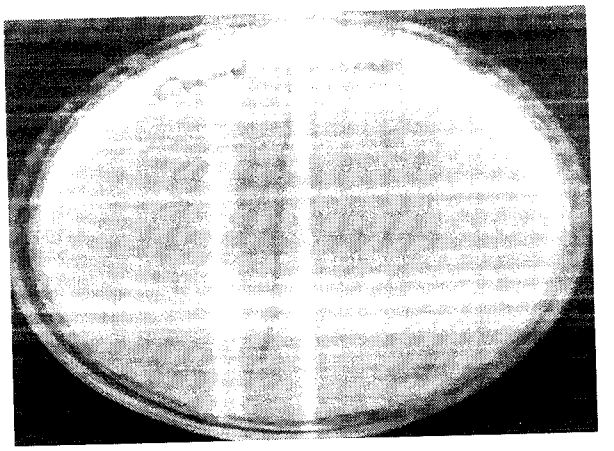
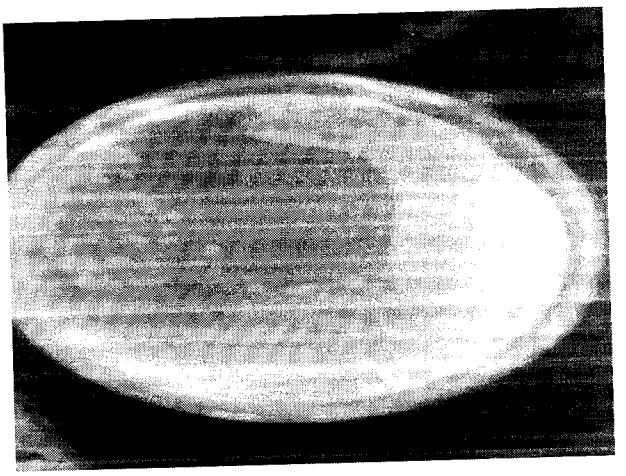


Figure-6:catalase activity

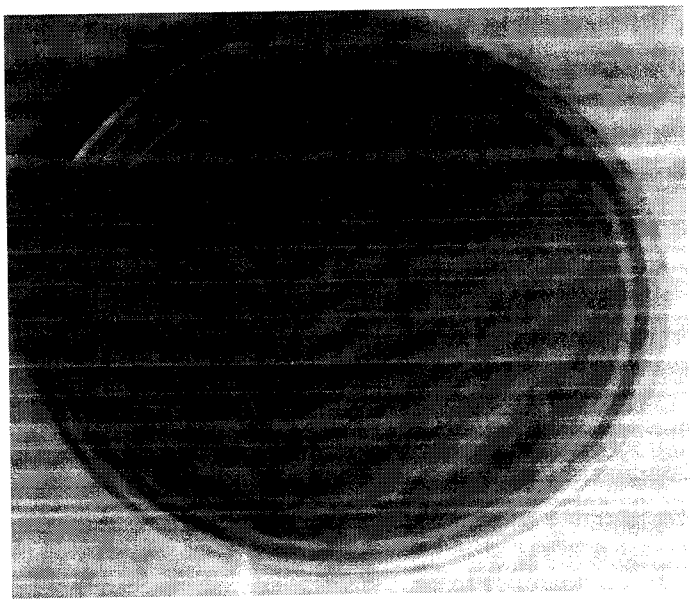


5.2 Degradation of feather by *Bacillus licheniformis*

Bacillus licheniformis was able to grow and produced keratinase in nutrient medium in which feather meal served as a additional carbon and nitrogen source and acted as enzyme inducer. resulted in nearly complete degradation of the feather after 7 days incubation at 40°C at 150 rpm. Keratinase activity was associated with growth at the maximum level of 10.76 U/ml. Kerotinolytic activity was measured in the absorbance at 280nm by the standard enzyme assay method

Figure-7: *Bacillus licheniformis* inoculated into nutrient broth Containing feather

Figure-7: *Bacillus licheniformis* grown on Feather Medium



5.3 Characterization of keratinase

The strain *Bacillus licheniformis* grew well and completely degraded poultry feather in the nutrient medium. The intense feather degrading was achieved in 40°C and initial pH adjusted to 7and 8. Similar growth curve were observed with in this range of temperature and pH.

5.3.1Effect of pH and its stability

The keratinase was active in neutral and alkaline condition with an optimum activity at pH 7 as shown in (Figure 8.) the enzyme activity with different buffers as shown in (Table 4) The enzyme was stable over a wide range of pH values, with the highest stability at pH 6-8 for 30 minutes as shown in (Figure 9.)

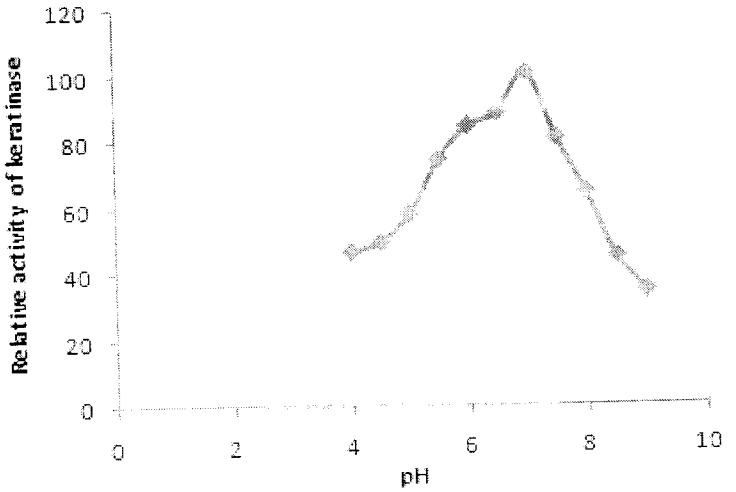


Figure 8. Effect of pH on keratinolytic activity

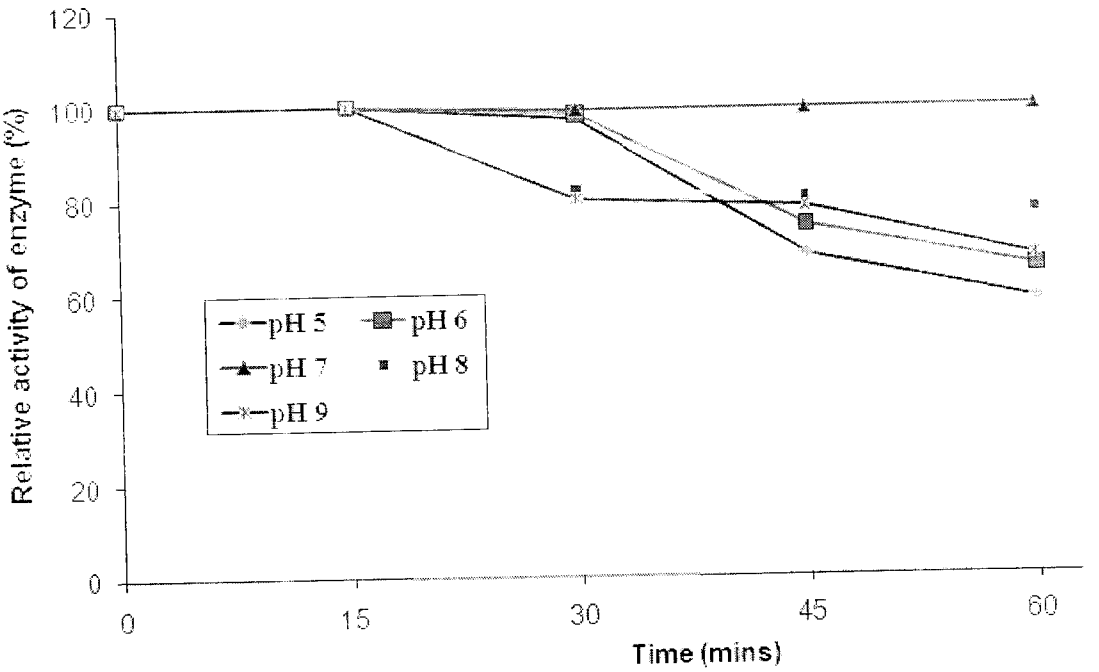


Figure 9. pH stability on keratinase from *Bacillus licheniformis*

TABLE 4. Effect of pH on keratinase activity

pH	Enzyme activity (U/ml)
4	9.65
4.5	10.21
5	12
5.5	15.36
6	17.44
6.5	18.21
7	20.71
7.5	16.65
8	13.21
8.5	9.21
9	7.11

5.3.2 Effect of temperature and its stability

The enzyme had an optimum activity at the temperature of 60°C and was rapidly inactivated at higher temperature. Above 80°C, the keratinase was no longer active. The enzyme was unstable at high temperatures but was stable at moderate temperatures as shown in (Figure 3.) The enzyme activity with different temperature as shown in the (TABLE.4)

Figure 10. Optimum temperature and temperature stability of keratinase from *Bacilluslicheniformis*:

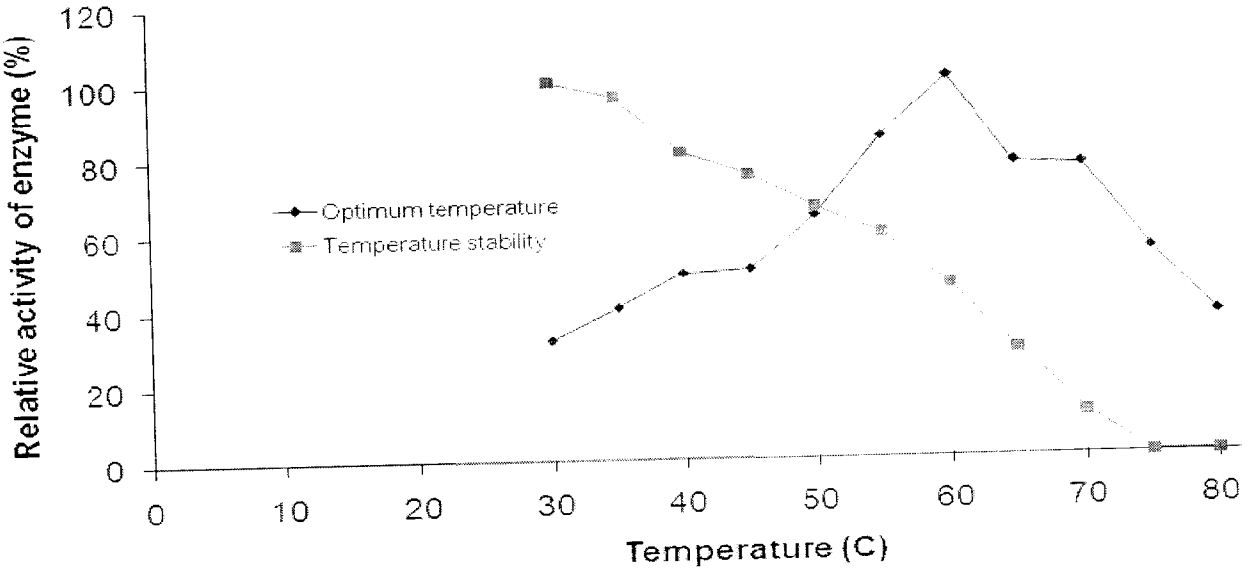


TABLE 6. Effect of Temperature on keratinase activity

Temperature	Enzyme activity (U/ml)
30	6.36
35	7.97
40	9.71
45	12.31
50	14.61
55	16.71
60	19.81
65	15.31
70	12.21
75	10.71

5.3.3 Effect of substrate concentration

The effect of substrate concentration on keratinase production was investigated. The affinities of the keratinase for keratin were determined at 60°C and pH 7.0 by a Line weaver Burk plot. The k_m and V_{max} values for keratin were 0.22 mg/ml and 0.01 U/ml respectively.

TABLE 7. Effect of substrate concentration on enzyme activity

Substrate concentration(mM)	V (U/mg)	1/s (mM^{-1})	1/v (mg/U)
0.1	39.6	10	0.0252
0.2	37.84	20	0.0264
0.3	44.96	30	0.0222
0.4	52.56	40	0.0190
0.5	54.72	50	0.0182
0.6	44.24	60	0.0226
0.7	29.6	70	0.0337

5.3.4 Effect of enzyme inhibitors and metal ions

Keratinolytic activity of keratinase was completely inhibited by EDTA, 1, 10 Phenanthroline by 83 and 94% respectively. This observation indicates that the crude keratinase belongs to the group of metalloproteases. Among the metal ions the Zn^{2+} and Mg^{2+} enhance the keratinolytic activity by 1.34 and 1.71 folds respectively and Cu^{2+}, Hg^{2+} and Cd^{2+} inhibits the keratinolytic activity by 92,94 and 93% respectively. The optimum activity was observed at 1mM as shown in (Table 6) and in (Fig 11 and 12)

TABLE 6. Effect of Inhibitors and Metal ions

	Concentration (mM)	Relative Activity (%)	Inhibition (%)	Enhancement
Crude	-	100	-	-
Zn^{2+}	1	134.02	-	1.34 folds
Mg^{2+}	1	171	-	1.71 folds
Hg^{2+}	1	5.86	94	-
Cd^{2+}	1	6.83	93	-
Cu^{2+}	1	7.18	92	-
1,10 Phenanthroline	1	5.48	94	-
EDTA	1	16.44	83	-

Results represent the average of three experiments

Figure 11. Effect metal ions on keratinase activity

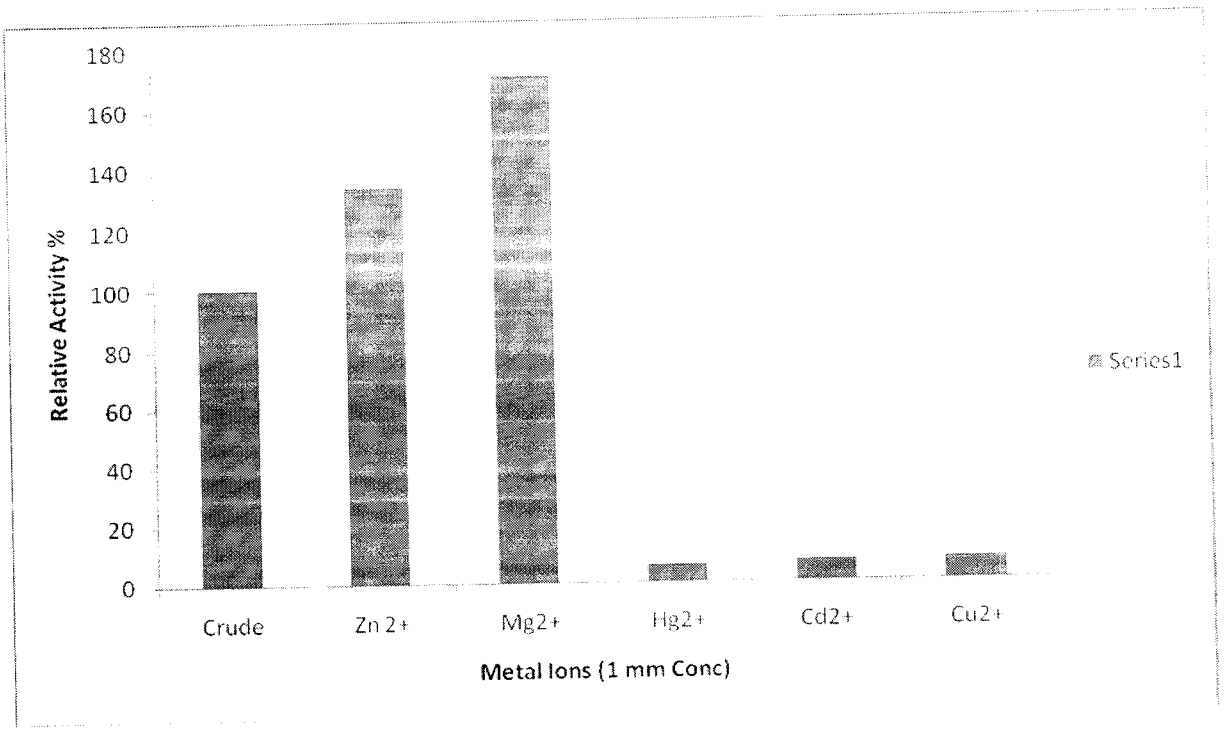
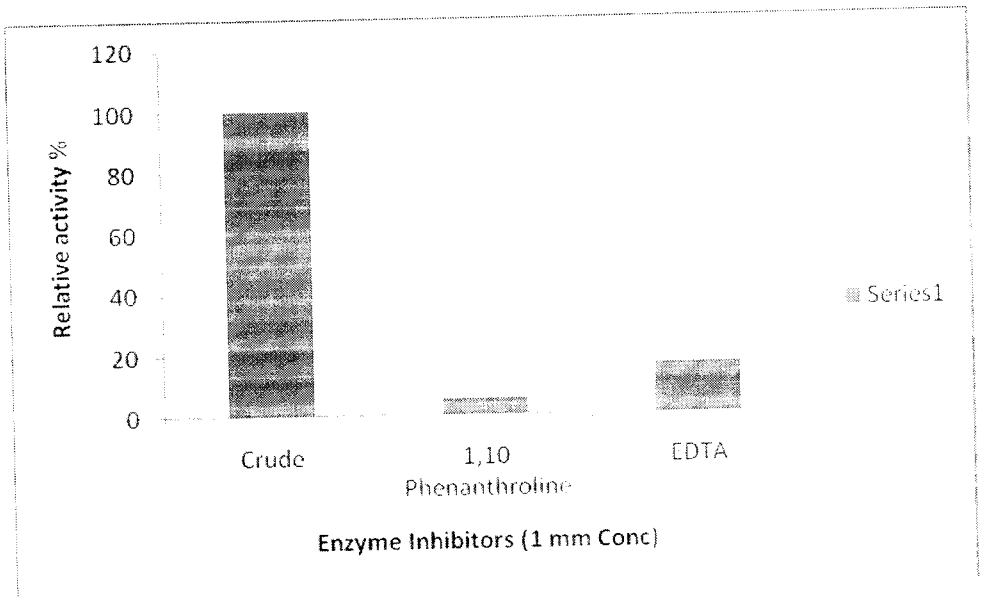


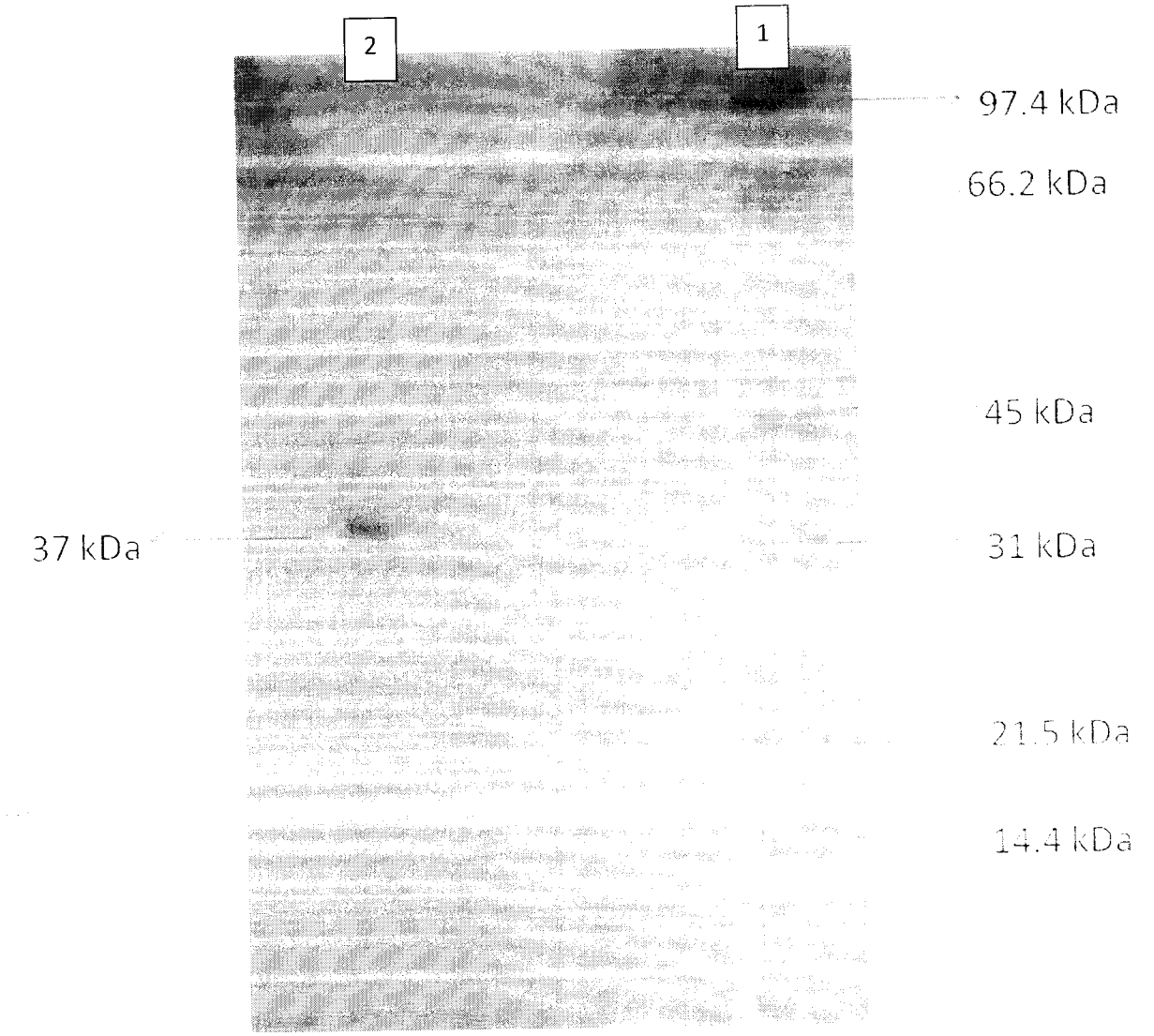
Figure 12. Effect enzyme inhibitor on keratinase activity



5.3.5 Keratinase purification

Keratinase from culture medium of *Bacillus licheniformis* was purified by 60% ammonium sulphate fractionation, followed by centrifugation pellet was dissolved in buffer and the dialysis was carried out. Then the sample was loaded in the column of DEAE-Sephadex ion exchange chromatography. These steps gave a purified keratinase with 13% yield and an purification fold of 12. The purified enzyme had a specific activity of 543 U mg^{-1} after the final step; A single protein band was shown on SDS-PAGE (Figure 13). The clear band was shown on SDS-PAGE indicating that the enzyme was purified to homogeneity.

Figure 13. SDS PAGE of Keratinase



Lane 1: Protein molecular marker(14 to 97 kDa)

Lane 2: Purified enzyme

TABLE 7. Purification table

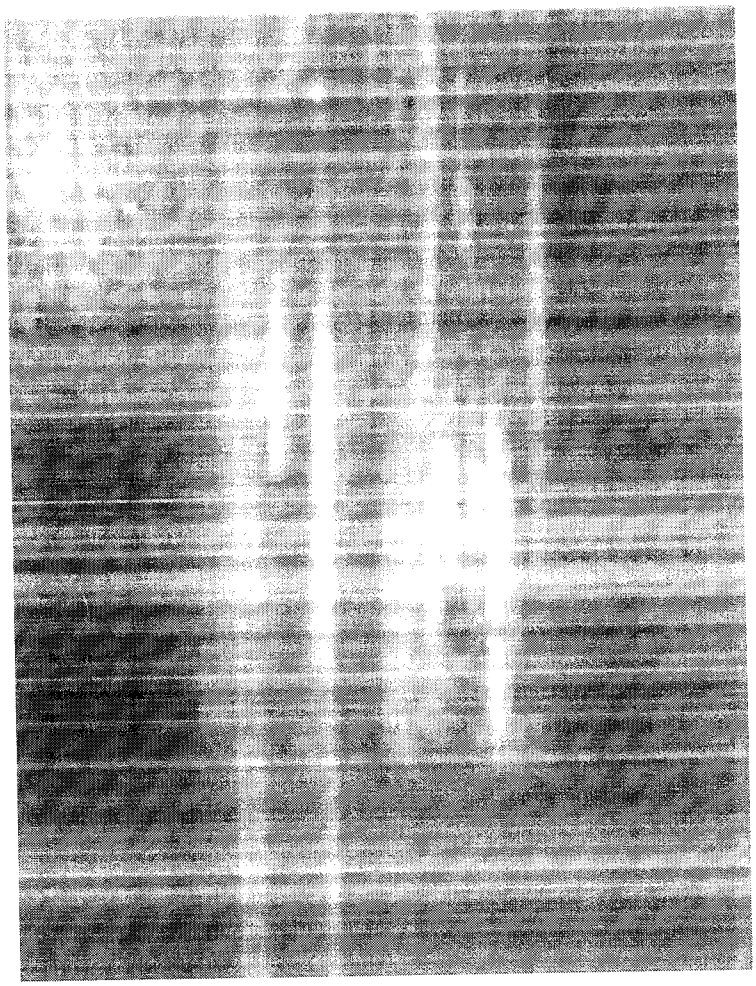
Purification steps	Volume	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/ml)	Purification fold	Yie (%)
Crude	250	10.72	2680	240	60	0.0446	1	1
Ammonium sulphate precipitation	35	15.52	543.2	45	1.575	0.3448	7.730	20.
Ion exchange chromatography	15	23.36	350.4	43	0.645	0.5432	12.179	13 4

5.3.6 Enzymatic feather degradation by weight loss method

Feather degradation was studied as a function of enzyme concentration (300–1500 U) followed by feather concentration (250 mg- 5 g) in 50 ml pH 7 buffer. It was observed that 543.25 U. keratinase are sufficient to completely degrade 1.5 g feather in 12 h.

Figure 14. Enzyme degradation of feather

A B



A. Control

B. After 12 hours treatment

5.3.7 Application of keratinase enzyme

The most important application of keratinase enzyme is that dehairing process in the leather and tannery industries.

The enzyme could improve the nutritional value of meat and poultry processing wastes containing keratin, collagen and gelatine. And it also acts as additive in poultry feed and in fertilizer, it may also serve as a source for amino acid.

This enzyme effectively degrade the feathers in poultry farm and it also degrade the horn meal

Discussion

6. DISCUSSION

The bacterium isolated from poultry waste has been shown to degrade feather keratin. The bacterium isolated from anaerobic habitat however, showed maximum growth under aerobic condition, as would be expected of a member of the family bacillaceae (William *et al.*, 1990). The optimal kerotinolytic activity was detected at 50°C, whereas previously described kerotinolytic bacteria mostly have feather-degrading activity in the temperature ranges from 40-60°C. *Bacillus* species have been reported to produce kerotinolytic protease (Kim *et al.* 2001; Savitha G. Joshi *et al.* 2007). The induction of keratinolytic enzyme produced by the species of *Bacillus* with feather powder, guinea pig hair human hair and nails, and cow horn and hooves was earlier reported (Cheng *et al.*, 1995; Lal *et al.*,1999 Tamilmani *et al.*, 2008). However, this strain isolated from namakkal soil able to degrade feather even at high temperature. The keratinase produced by *Bacillus licheniformis* was induced by the presence feather meal. The presence of this species in a poultry waste may be that the bacterium is indigenous to the chicken gut. However, more likely that, it was indigenous to the environment in which poultry excreta are collected. The environmental also contain feathers and the isolate may have adapted to utilize this substrate. The most studied kerotinolytic bacteria are *Bacillus licheniformis* which have been described to possess feather degrading activity. Micro organism growing on nutrient medium containing feather meal also act as carbon and nitrogen source Substrate level in the medium may regulate enzyme secretion. *Bacillus licheniformis*, showed to be more adapted to keratinase production using keratin as substrate since maximum kerotinolytic activity of the isolate was observed during early growth, and the strain displayed a higher enzyme activity during incubation.

The enzyme activity was studied over a broad range of temperature (30-80°C). The optimum temperature of keratinase from *Bacillus licheniformis* 60°C was slightly higher than that of other bacillus kerotinolytic protease (50-55°C) (Cheng *et al.*1995; Balaji *et al.*2008). The enzyme was stable at high temperatures partly because its catalytic site was well protected by a substrate. Further increase in the temperature to 80°C reduced the activity. The active range of pH 5.0 and 7.0 whereas the optimum pH for the keratinase was found to be at pH 7.0, The enzyme was stable at the range of 6.0-8.0 (Cheng *et al.*1995). the activity decreased at pH 3.0 and 8.0. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperature (Lin *et al.*1999) and some mesophilic bacteria exhibit the optimal [illegible] ranging from 20 to 30°C (Allpress *et al.* 2002). Here the

activity continued beyond ambient temperature. The Keratinase from *Bacillus licheniformis* is very likely to be a serine protease since it was mostly inactivated by 1, 10 phenanthroline and EDTA and its pH optimum was in a basic range. Several reports have shown serine protease to be slightly affected by metalloprotease inhibitor (Balaji *et al.*2008). Divalent metal ion activated the keratinase activity of *Bacillus licheniformis* For Keratinase purification, from the crude enzyme protein precipitation with ammonium sulphate it gave low yield and increases the purity..

After the enzyme had been passed through ion exchange column it did not attach to either type of media indicating that the enzyme had no net charge under the condition investigated. This is unusual behavior in respect of keratinolytic protease purification. It may be because, under the purification condition, the enzyme formed non-polar amino acid residues outside its structure which could be comparable to hydrophobic structure of keratin (substrate).

It was also observed that keratinase inhibitors or contaminants interfering with the activity assay could be removed by ion exchange chromatography as seen by the increased enzyme activity and yield as in the report of (Lin *et al.*1992).

Enzymatic degradation of feather is noteworthy as pure keratinases from other bacterial strains wich are reported to degrade 10–20% feather in absence of live or chemical redox mediator Such an enzymatic process for feather meal production would be cost effective and simpler method over bacterial fermentation (Ramnani and Gupta, 2007) which needs several controls during bioreactor run.

The molecular weight of keratinase from *Bacillus licheniformis* (37kDa) was similar to that of *Bacillus licheniformis* PWD-1, Steptomyces pactum DSM 40530 and *Doratomyces microspores* (30-33kDa) (Lin *et al.*1992;Gradisar *et al.* 2000). However different molecular mass was found at 18kDa of *Streptomyces albidoflavus* and at 40 kDa of *Steptomyces thermoviolaceus* (Chitte *et al.* 1999)

Therefore, this enzyme could be applied to digest keratin substrate under various metal ion surroundings. The enzyme showed partial on chicken feather and hair since these substrates contained disulphide linkages which are a crucial structural feature of their molecules. This result indicates that this enzyme could not completely cleave only peptide

human hair by physical method or reducing agents, or detergents, or activation of the enzyme by adding metal salts are required for improvement of their degradation. Keratinase produced by *Bacillus licheniformis* plays part feather degradation. The bacterium and its enzyme could be used to improve nutritional values of animal feed containing feather, or keratin, or poultry processing waste in namakkal region.

7. CONCLUSION

- The bacteria, *Bacillus licheniformis* was found to produce the enzyme keratinase
- It has been cultured to purify the enzyme
- The purification steps includes ammonium sulphate precipitation, ion exchange chromatography and SDS PAGE
- About 12.17 fold purification was obtained
- The crude enzyme was characterized, its optimum pH was found as 7.0, optimum temperature was 60°C and its km value is obtained as 0.22 mg/ml and in metal ions zinc and magnesium increases the keratinase activity of 1.34 and 1.71 folds and mercury cadmium copper inhibit the keratinase activity. The EDTA and 1,10 phenanthroline inhibit the keratinase activity.
- It was observed through weight loss method at 543.25 U of keratinase are sufficient to completely degrade 1.5 g feather in 12 h.
- The application of keratinase was studied with Bioconversion of feather with *Bacillus licheniformis*, has potential to protect our environment. This novel keratinolytic isolate could be a potential candidate for the degradation of feather keratin and also in de-hairing process in leather industry. And also act as additives in poultry field.
- This result obtained were clearly shown that this is cheap source if it used in poultry and leather industry
- The large scale production of this enzyme can be done.

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Appendices

9. APPENDICES

Appendix I

Phosphate Buffer composition

Stock solutions:

A: 0.2 **M** solution of monobasic sodium phosphate (27.8g in 1000mL)

B: 0.2 **M** solution of dibasic sodium phosphate (53.65g of $Na_2HPO_4.7H_2O$ or 71.7g of $Na_2HPO_4.12\ H_2O$ in 1000mL)

xmL of *A*, ymL of *B*, diluted to a total of 200mL.

x	y	pH
87.7	12.3	6.0
85	15	6.1
81.5	18.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0

Appendix II

Estimation of protein by Lowry's method

Reagents

- Stock standard: Bovine serum albumin
- Dissolve 100mg of BSA and make upto 100ml with distilled water.
- Working standard solution:
- Take 10ml of stock standard and dilute to 100ml with distilled water.
- 2% sodium carbonate in 0.1N sodium hydroxide (Reagent A)
- 0.5% copper sulfate in 1% potassium sodium tartarate (Reagent B)
- Alkaline copper solution
- Mix 50ml of A and 1ml of B prior to use
- Folin coicalteau reagent (1:1) should be freshly prepared.

Appendix III

Feather media composition (g/l):

Yeast extract	-	3.0
Feather powder	-	2.0
Vitamins	-	1.0
Agar-Agar	-	2.0
Salt	-	2.0
pH	-	7.5 – 8.0

The culture was incubated at 37°C for 2 days.

Appendix IV

Activity (U/ml) = $4 \times n \times A_{280}/(0.01 \times 10)$

Where,

n = Dilution rate,

4 = Final reaction volume (ml)

10 = Incubation time (min)