CHICKEN FEATHERS AS AN ALTERNATIVE SUBSTRATE FOR EXTRACELLULAR KERATINASE PRODUCTION USING *Microsporum fulvum* IBRL SD3 BY SOLID SUBSTRATE FERMENTATION

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by

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TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
°C	Degree Celcius
g	Gram
g	Gravity
K	Kilo
M	Molar
U	Unit
µ	Micro
cm	Centimeter
mm	Milimeter
µm	Micrometer
nm	Nanometer
Kg	Kilogram
mg	Miligram
µg	Microgram
µl	Microliter
kDa	Kilo Dalton
bp	base pair
rpm	Revolution per minute
Rf	Relative mobility
v/v	volume over volume
w/v	weight over volume
w/w	weight over weight
BSA	Bovine serum albumin
CMC	Carboxymethyl cellulose

DEAE	Diethylaminoethyl
PDA	Potato dextrose agar
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSF	Solid substrate fermentation
SmF	Submerged fermentation
SEM	Scanning electron microscope
TEM	Transmission electron microscope

BULU AYAM SEBAGAI SUBSTRAT PILIHAN UNTUK PENGHASILAN KERATINASE EKSTRASEL MENGGUNAKAN *Microsporum fulvum* IBRL SD3 SECARA FERMENTASI SUBSTRAT PEPEJAL

ABSTRAK

Di alam semulajadi, bulu ayam telah dihasilkan dengan banyak daripada industri penternakan ayam dan ia mengakibatkan masalah kepada persekitaran. Keupayaan degradasi oleh mikrob dan biopenukaran bulu ayam adalah matlamat utama kajian ini dijalankan. Dalam kajian ini, bahan buangan berkeratin digunakan sebagai substrat untuk menghasilkan enzim keratinase oleh *M. fulvum* IBRL SD3 yang telah dikenalpasti secara molekular melalui fermentasi substrat pepejal. Penambahbaikan keadaan pengkulturan untuk menghasilkan keratinase di dalam sistem kelalang goncangan adalah dengan mengunakan 0.75 mm saiz zarah substrat, 100% (w/w) kandungan kelembapan awal, suhu bilik (30±2ºC) sebagai suhu pengeraman, pH 7 sebagai pH awal, pengadukan sekali pada setiap 24 jam dan saiz inokulum sebanyak 1 X 10^7 spora/ml meningkatkan penghasilan keratinase sehingga 0.266 U/g substrat terfermentasi pada hari ke 6 pengkulturan. Dalam penambahbaikan keadaan komposisi medium pula, penambahan sumber karbon tidak diperlukan, hanya sedikit penambahan iaitu 0.70% (w/w) ekstrak yis diperlukan untuk menghasilkan aktiviti keratinase yang maksimum pada hari ke 6 pengkulturan sebanyak 0.372 U/g substrat terfermentasi dengan kenaikan sebanyak 905.41% berbanding profil awal. Kemudian, penambahbaikan sistem dulang dijalankan dengan menggunakan dulang aluminium cetek yang berukuran 16 cm x 16 cm x 5 cm. Penghasilan keratinase

optimum didapati pada hari ke 6 pengkulturan dengan 1.065 U/g substrat terfermentasi dengan parameter optimum pada 1.00 cm ketebalan substrat, 100% (w/w) kandungan lembapan awal, pengadukan sekali pada setiap 24 jam dan saiz inokulum sebanyak 1 X 10^7 spora/ml. Aktiviti keratinase meningkat pada 2878.38% berbanding penghasilan aktiviti keratinase pada komposisi medium dalam kelalang goncangan. Seterusnya keratinase kasar ditulenkan melalui kromatografi penukaran anion dan penurasan gel lalu dielektrofikasi melalui SDS-PAGE memberi keputusan berat molekul 153.03 kDa. Keratinase tulen kemudiannya dicirikan dan mencapai suhu optima pada 50ºC dan stabil pada suhu 37ºC. Keadaan pH adalah optimum dan stabil pada pH 8. Keratinase tulen berupaya menghidrolisis kasein dan albumin serum bovin (BSA) berbanding keratin asli seperti sisik ikan, rambut dan kuku. Keratinase tulen direncatkan dengan kehadiran ion Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+} dan EDTA. Pembiodegradan substrat oleh *M. fulvum* IBRL SD3 dapat dilihat melalui pemerhatian mikroskopik menggunakan SEM dan TEM.

CHICKEN FEATHERS AS ALTERNATIVE SUBSTRATE FOR EXTRACELLULAR KERATINASE PRODUCTION USING *Microsporum fulvum* IBRL SD3 BY SOLID SUBSTRATE FERMENTATION

ABSTRACT

In nature, chicken feathers were abundantly generated from poultry industry and become severe environmental problems. The capabilities of microbial degradation and bioconversion of chicken feathers were directed to the reason for this study. The present study used the keratinaceous waste as a substrate in keratinase production from molecular identification of *M. fulvum* IBRL SD3 via solid substrate fermentation. The improvements of cultural conditions for maximal keratinase production in a shake flask system with particles size of substrate of 0.75 mm, at the initial moisture content of 100% (w/w), cultivation temperature of room temperature (30±2ºC), initial pH 7, mixing frequency at once every 24 hours and inoculum size of 1 X 10^7 spores/ml showed keratinase achieved 0.266 U/g of fermented substrate on day 6^{th} of cultivation. In the improvement of medium compositions, no additional carbon source was required and a slight supplementation of 0.70% (w/w) of yeast extract to produce the maximum keratinase activity on day 6^{th} of cultivation at 0.372 U/g of fermented substrate with the increment of keratinase activity at 905.41% compared to the initial profile. Furthermore, an improvement of tray system was conducted using a shallow aluminium tray (16 cm x 16 cm x 5 cm). The optimum of keratinase yield was obtained on day 6^{th} of cultivation with 1.065 U/g of fermented substrate with optimal parameters using 1.00 cm of substrate bed height thickness,

initial moisture content of 100% (w/w), mixing frequency at once every 24 hours and inoculum size of 1 X 10^7 spores/ml. Keratinase activity increased 2878.38% compared to keratinase production in the improvements of medium compositions in shake flask system. Consequently, crude keratinase was purified using anion exchange and gel filtration chromatography thus electrophoreted using SDS-PAGE resulted in 153.03 kDa of molecular weight. The purified keratinase was further characterized and the optimal temperature was 50ºC and temperature stability found at 37ºC. The optimum and stability of pH was at pH 8. The purified keratinase was capable to hydrolyzed casein and bovine serum albumin (BSA) in comparison with fish scales, hair and nail. The purified keratinase was inhibited by the presence of Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Zn^{2+} dan EDTA. The substrate biodegradation by *M. fulvum* IBRL SD3 occurrence was substantiated by microscopic observation using SEM and TEM.

CHAPTER 1

INTRODUCTION

1.1 Poultry waste and environmental issue

Livestock is one of the key areas of the agricultural sector, which contributes billions worth of trade to the economy. In Malaysia, poultry industries are the most commercialized and integrated livestock sector with the support of the government into position Malaysia as a major world food exporter (Ministry of Agriculture and Agro-based Industry Malaysia). There are drastic increments in a poultry population from 2003 to 2008 with up to about 216 million poultry populations are produced (Agriculture Statistical Handbook, 2008**)** to meet the increasing demand due to increasing in population, economic growth and lifestyle changes. Unfortunately, in growing of the livestock sector, the wastes generated from this industry are left in an undesirable's manner to the environment.

Each year, million tones of chicken feathers are produced as a waste from commercial poultry processing industries. Chicken feathers consist of 90% protein whereby the main component is keratin (Gessesse *et al*., 2003), which makes it hard to be degraded in nature. Therefore, the major concern is how to manage the waste from our local poultry processing industries. Most of the poultry plant or chicken broiler farming use conventional method such as burning and disposing them in the garbage disposal dumps. The disadvantages of the conventional methods are the slow

rate of decomposition of the waste which produces foul smell, production of greenhouse gases and some of the waste being channeled into surrounding rivers which contributes to pollution. The amount of solid waste in Malaysia has steadily increased and the government is still looking for the best method to overcome this problem. The environmental awareness has also risen amongst Malaysian as it can be seen in the solid waste management where it is a priority area under the Ninth Malaysian Plan.

1.2 Biotechnology: A promising method

Chicken feathers contain α-and β-helices keratin structure which makes it hard to be degraded by well known proteases such as pepsin, tripsin and papain (Papadopoulos, 1986). The recalcitrant being formed due to the high degree of cross linkage of disulphide bonding, hydrogen bonding and hydrophobic interaction (Ignatova *et al*., 1999; Marcondes *et al*., 2008). Therefore, the accumulation of these undegradable chicken feathers has led to the environmental issue if it is not prevented.

Recently, an alternative method exploiting the capability of microorganisms to degrade the keratin has been devised (Bertsch and Coello, 2005). The biotechnological impetus has been gained in hydrolyzing keratin from chicken feathers into soluble protein and rare amino acid. These keratinolytic microorganisms such as bacteria, fungi and acetomycetes are widespread in nature and can be used to

degrade the keratin (Onifade *et al*., 1998). Chicken feathers can be used as a substrate for fermentation and the protein from the feathers can be formulated into animal feed as it is high in amino acid such as cysteine, valine and treonine. Besides, it can replace the soy bean meal used in animal feed formulation (Apple *et al*., 2003). Biotechnological approach involving enzyme production using microbial activities have been proven to be efficiently in providing a low cost and can also upgrade the nutritional value and environmental friendly (Onifade *et al*., 1998).

This enzymatic biodegradation has played a prominent role in transforming "waste to wealth" and attracted a lot of scientists in the recent decade, particularly due to its multitude applications in industries such as in animal feed, fertilizer, leather, pharmaceutical, detergent, and renewable bioresources (Gupta and Ramnani, 2006).

1.3 Research objectives

Poultry waste from livestock sector can be converted to various additional valued products. Thus, this study focuses on the use of poultry feathers to produce keratinase enzyme and protein meal via solid state fermentation. Besides, none of essential application utilizing chicken feathers in term of enzyme production has been documented in Malaysia. However the potential of enzymatic biodegradation has been proven successful in the outside world and this research was undertaken to promote the use of waste material in order to acquire keratinase and help conserve the environment.

The objectives of this study were;

(1) To improve the cultural conditions and medium compositions for maximum keratinase production under solid substrate fermentation in shake flask and shallow tray systems
(2) To purify and characterize the keratinase enzyme
(3) To study the degradation process of feathers by the fungus

CHAPTER 2

LITERATURE REVIEW

2.1 Fermentation

The term of fermentation is originated from Latin verb *fevere,* means to boil. Fermentation is one of the oldest constitutions of food preservation technologies in the world. Fermentation precedes human history, has denoted that it has been practiced during ancient Egypt with beverages were fermented in Babylon circa 5000 BC (Dirar, 1993). However, it has different meanings to biochemist and industrial microbiologist. Biochemically, it is related to generation of energy by the catabolism of organic compounds, whereas it carries a much more extensive definition in industrial microbiology as to describe any process for the production of the product by the mass culture of microorganisms (Stanbury *et al*., 1995). Certainly, the development of fermentation had revolutionized and demand for it is likely to increase due to its advent contributions on various biotechnological aspects. Production of microbial cell or biomass as the product, production of microbial enzymes, production of microbial metabolites, production of recombinant products and modification of compounds which are added to fermentation or transformation process are the five main groups of commercially important fermentations (Stanbury *et al.*, 1995).

2.1.1 Solid substrate fermentation

Solid substrate fermentation can be defined as the growth of microorganisms on solid material or substrate that act as a carbon or energy source in the absence or near absence of free water (Pandey *et al*., 2001) wherein resembles the microorganisms adaptation in natural environment (Hölker *et al*., 2004). Meanwhile, solid state fermentation can be explained as a fermentation process that utilizes solid natural substrate or an inert substrate used as a solid support in the absence or near absence of free water (Pandey *et al*., 2001). This is a substantially different compared with submerged fermentation where the aqueous phase is the main element in the fermentation process. Recently, solid state and solid substrate fermentation have shown biotechnological impetus and has been employed in many areas in bioprocess such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops for nutrient enrichment purposes, bio-pulping and several other value added products such as enzymes, organic acid productions, biosurfactants, biopesticides and biofuel (Pandey *et al*., 2000). Since the development of solid substrate fermentation has been evolving rapidly and the process is understood, the production has been implemented in larger scale such as in industrial scale. For example, a traditional Koji production in Japan uses steamed rice as a solid substrate inoculated with solid strains of the filamentous fungus *Aspergillus oryzae* (Liang *et al*., 2009; Chancharoonpong *et al*., 2012) to produce and preserve foods in order to enhance the flavour of the ingredient and to increase its nutritional value while at the same time make it less perishable. It has now very important in Japan's food manufacturing industry. The upshot has been

far out of home cooking and excessive dependence on the food service industries (Fujita, 2008).

2.1.2 Advantages and disadvantages of solid substrate fermentation

Both fermentation systems differed on several characteristics like substrate size, water usage, aeration, speed of agitation, scale-up process, energy consumption, the risk of contamination and capital investment. These significant characteristics confer the advantages and disadvantages for either solid substrate fermentation or submerged fermentation. The fundamental knowledge of fermentation is a prerequisite for selecting any desired fermentation system for further optimization studies. Microorganisms in solid substrate fermentation are under closer conditions of the natural habitats, therefore they probably can afford to produce a certain product which cannot be produced or restrictedly produced in a submerged culture (Szewczyk and Myszka, 1994). These advantages vindicate the reason of revival activities and the prominence of solid substrate fermentation as a significant method for microbial conversion product.

Nowadays, solid substrate fermentation has become more attractive compared with submerged fermentation caused by reactor modification and technological improvements. There are four existing reactors which impersonated the best natural ways of performing solid substrate fermentation. All of the bioreactors can be differentiated according to aeration and mixed system engaged. The most basic

bioreactor is a tray system in which using a flat tray with a thin layered substrate has been distributed (Couto and Sanroman, 2006), packed bed system consists of glass or plastic column that retained the solid substrate on a perforated base with air pre-humidification (Bellon-Maurel *et al*., 2003; Kumar and Jain, 2008), horizontal drum which allowed enough aeration and mixing of the substrate in a vessel using paddles or baffles (Hardin *et al*., 2002; Prado *et al*., 2005) and fluidized bed that supplied continuous agitation with forced air (Wang and Yang, 2007). A distinguished advantages and disadvantages in all those bioreactors had motivated to a new developing bioreactors configuration and modification (Susana and Sanroman, 2005).

Referring to Table 2.1, the advantages of solid substrate fermentation are more apparent than its disadvantages. In most of solid substrate fermentation process, the product titers are higher compared with its waste water produced in downstream processing, which indicates that it requires less water in upstream process and thus reduced the downstream processing costs. Enzyme titers are higher in solid substrate fermentation than in submerged fermentation when compared with the same strains and using the same fermentation broth (Viniegra-Gonzales *et al*., 2003). Low moisture conditions needed in the process also support contamination reduction. There is no complicated design of bioreactors and agro-industrial residues used for solid substrate fermentation, hence, it is more economical. Moreover, in the absence of severe mixing, there is no foam formation that occurred which usually admitted in submerged fermentation.

Table 2.1 The advantages and disadvantages of solid substrate fermentation process

Advantages	Disadvantages
• Higher product fibers • Lower capital expenditure • Lower waste water output (less water needed) • Reduce energy requirement • Absent of foam formation • Simplicity of medium growth • High reproducibility • Simple fermentation media • Less fermentation space • Absence in rigorous control of fermentation parameter • Easier aeration • Economical to use even in small scale • Easier contamination control • Applicability of using fermented solid directly • Storage of dried fermented matter • Lower costs of downstream processing	• Difficulties in controlling the physical parameters • Problems with development of heat during the fermentation process • Difficulties in scaling up technique

[Adapted from Stanbury *et al.*, (1995); Pandey *et al*., (2001); Susana and Sanroman (2005)]

2.2 Keratin

Keratin is a fibrous protein found in vertebrates and conferred protective and structural functions which generally contains large quantities of sulphur-containing amino acids, particularly cystine (Böckle and Muller, 1997; Vignardet et al., 2001; Shankar *et al*., 2010). Cystine ($C_6H_{12}N_2O_4S_2$) derived when two monomers of cysteine ($C_3H_7NO_2S$) were oxidized (Figure 2.1). Keratinaceous material is a major component of feathers, hair, hoofs, horns, nails, scales, scalps, *stratum corneum*, and wools (Vignardet *et al*., 2001). However, the indigenous state of keratin cannot be degraded by commonly known proteolytic enzymes like papain, pepsin and trypsin due to its high mechanical resistance of its polypeptide chain (Papadopoulos, 1985). Unique characteristic of keratin hinges of its structural configuration existed in this tight folding of the supercoiled protein chain in α-helic (α-keratin) and β-sheets (β-keratin) manifested by the strong association of disulphide bonding (Kreplak *et al*., 2004; Anbu *et al*., 2005; Fraser *et al*., 2008). The keratin fibrils in both conformations are distorted into microfibrils that justify the stability and withstand the biological degradation by enzymes (Kreplak *et al*., 2004; Zerdani *et al*., 2004). However, keratin can be degraded by some microorganisms capable of producing keratinase. This enzyme can hydrolyze keratin into smaller peptide and thereupon can be absorbed by the cells (Marcondes *et al*., 2008). Keratin is classified into two; hard keratin (5% sulphur) and soft keratin (1% sulphur) depending on its sulfur content. Hard keratin is more rigid and usually existed in appendages like feathers, hair, hoofs and nails which contain high disulphide bond whereas soft keratin can be found in the epidermis and callus. Soft keratin has low content of disulphide bond, which make it more pliant and flexible (Voet and Voet 1995; Schrooyen *et al*. 2001).

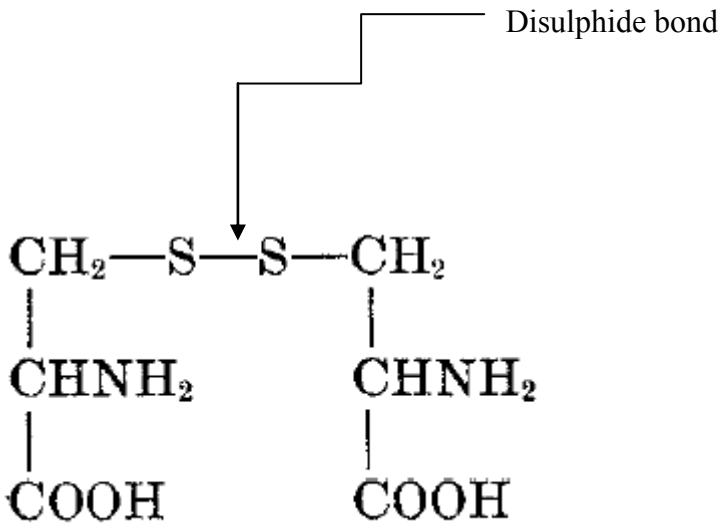

Figure 2.1 **Cystine formation by oxidation of two cysteine residues that covalently linked to form disulfide bonds (Butz and Du Vigneaud, 1932).**

2.2.1 Feathers topography

Feathers are one of a prominent element features in avian anatomy and evolved from scale (Raptor Research Foundation, 2012). It has strong and flexible structure. Feathers provide thermoregulation through insulation and maintained the body temperature at around 40ºC for most of the birds apart from allowing birds to fly. A typical wing feather (Figure 2.2) consists of a central stiffer supporting shaft called the rachis, with the softer vanes on each side which lead the edge of feather during flight called the outer vane. The opposite vane is wider than the outer vane and is referred to as the inner vane. The side branches are called barbs and are linked together by a set of barbules and their hooklets are sometimes called hamuli. The calamus or quill is the base of the feather. It is hollow and there are no side branches. The inferior umbilicus is embedded into the skin, connecting bloods and growing feathers of birds. In feathers, keratin exists in the beta sheet configuration which composed of hydrogen bond protein strands into beta pleated sheets and further twisted and cross linked by disulphide bridges and turn out to be more rigid than alpha keratin of mammalian keratin materials. Studies of X-ray diffraction verify the presence of helical filaments consist of repeated units in feathers. Filaments found in avian feathers and reptilian scales make up of a pair of twisted beta sheet domains, each composed by a 23 residues (Fraser and Parry, 2008). Generally, the physical and mechanical properties of feather keratins are strongly influenced by their shape and makes keratin highly resistant against physical, chemical and biological agents (Lynch *et al*., 1986). Due to its desired properties as light and waterproof, recently there are available product manufactured using feathers in thermal insulation, automotive industry, paper alternatives, biodegradable composites, diaper filling,

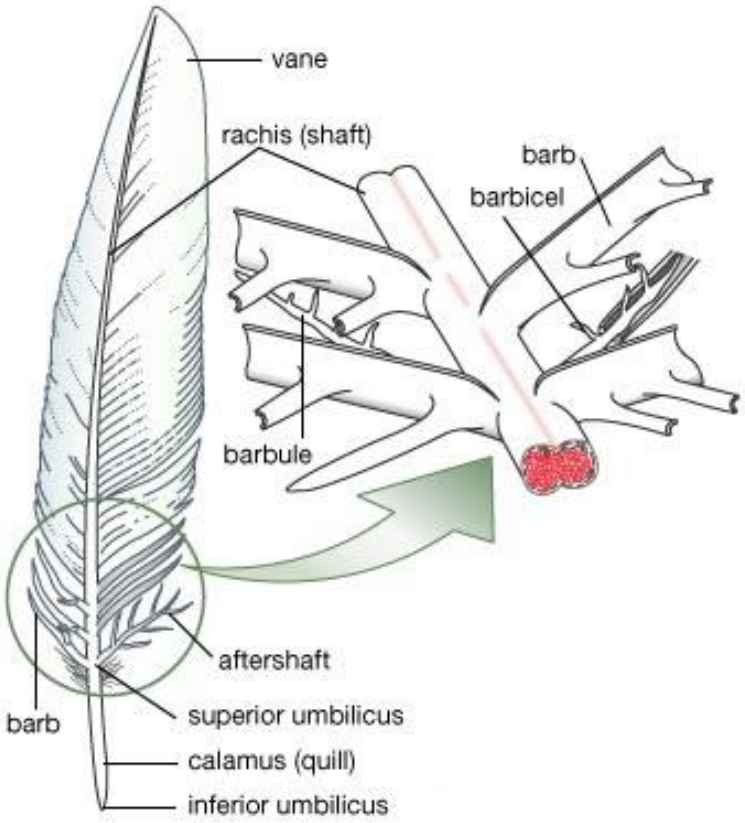

Figure 2.2 Feather topography

Source: [http://www.meriam-webster.com]

water filtration fibers and biodegradable pseudonylon fabrication (Blicq, 2010).

2.2.2 Chicken feathers

Commercially, world-wide poultry processing plants generate million tons of feathers every year which consisting approximately 90% of keratin and like another form of keratin they are slow in their decomposition. Feathers number was estimated between 7000 and 9000 in an adult chicken together with feather weight at 3-6% of chicken body weight (Leeson and Walsh, 2004). Several considerable variability of amino acid for feathers have been reported by a few researchers to date (Graham *et al.,* 1949; Block and Weiss, 1956; McCasland and Richardson, 1966; Fisher *et al.,* 1981; Stilborn *et al.,* 1997). Table 2.2 show that broiler chicken feathers contains many essential amino acids, and the amount of amino acid released increased as the degradation days increased.

According to Fisher *et al*. (1981), amino acid content in chicken feathers was consistent prior to time with minor depletion in methionine and increasing in the threonine, valine and leucine content. These amino acids play an important role in the growth performance of broilers (Pinto *et al*., 2003; Zhan *et al*., 2005; Silva Junior *et al*., 2006). Considering this, many researchers and manufacturers are applying and converting waste chicken feathers into valuable and nutritious by product such as feather meals replacing the widespread market of soybean meals.

Currently, the conversion of feathers to feather meal used conventional method involving physical and chemical treatments. A lot of treatments have been developed

to increase high digestibility of feather meal and categorized into two groups: hydrothermal treatments and microbial keratinolysis (Onifade *et al*., 1998). Hydrothermal treatments usually engage high temperatures (Wang and Parsons, 1997) or high pressure, with the addition of strong acids like hydrochloric acid (Eggum, 1970), or alkaline such as sodium hydroxide (Papadopolous, 1985). Without suitable processing, nutritive value of essential amino acid in feather meal can be degraded, after cooking at high temperature, the digestibility of the treated feather meal was 16% lower than the excessive insoluble fraction collected after the process (Wang and Parson, 1997).

An alternative method that can be used to improve feather digestibility is biodegradation by keratinolytic microorganisms, therefore, it is an environmentally friendly biotechnological process. Myriad of microorganisms which include bacteria, fungi and actinomycetes are found capable to degrade keratin in nature and able to produce keratinases and peptidases (Mazotto *et al*., 2011). Keratinophillic microorganisms that have been reported to be used in microbial keratinolysis treatments are *Bacillus licheniformis* (William *et al*., 1990) *Microsporum gypseum* (Page and Stock, 1974) and *Streptomyces pactum* (Böckle *et al*., 1995).

2.3 Keratinase

In accordance with keratinase characteristic, the Nomenclature Committee on the International Union of Biochemistry in 1978, keratinase is recommended as a proteolytic enzyme and is classified as proteinase of unknown mechanism with enzyme commission number (EC 3.4.99) in enzyme nomenclature (Gupta and Ramnani, 2006). Yet, several researchers categorized keratinase as a serine protease because it's highly equal to 97% of sequence homology with alkaline protease. Keratinase is inhibited by the serine protease inhibitors (Bressollier *et al*., 1999). Keratinase enzyme can hydrolyze keratin into smaller particle that can be absorbed by cells by breaking the disulphide bond. The purified keratinase enzyme from the class of serine protease and metalloprotease have high proteolysis activity against insoluble keratinaceous materials such as feather, hair, nails, hoof, and scale which are hardly degraded. Keratinase is commonly active outside the cell where it is transported out from the intracellular synthesis site. However, *Trichophyton mentagrophytes* and *Trichophyton rubrum* secrete out the proteinase associated with cell (Yu *et al*., 1971; Lamkin *et al*., 1996). Determination of keratinase molecular weight has been extensively studied. The molecular mass range is between 18 kDa and 440 kDa and it is variable depending on microorganisms (Gupta and Ramnani, 2006; Yu *et al*., 1971). Keratinase enzyme secreted from an actinomycetes, *Streptomyces albidoflavus* holds molecular weight at 18 kDa (Bressollier *et al*., 1999) meanwhile, an exocellular keratinase produced by a Gram positive bacteria; *Kocuria rosea* has a molecular weight of 240 kDa (Bernal *et al*., 2006).

Table 2.2 Amino acid content in broiler chicken feathers

	Age (days)				
	14	28	42	56	84
Protein (%)	93.9	91.2	95.7	93.4	94.6
Amino acid (%)					
Arginine	6.8	6.4	6.8	6.4	7.0
Cystein	7.5	7.9	7.2	6.8	7.7
Histidine	1.4	0.7	0.6	0.6	0.5
Isoleucine	4.3	4.5	4.6	4.6	4.8
Leucine	7.8	7.7	7.9	7.8	8.3
Lysin	3.0	1.9	1.9	1.7	1.6
Methionine	1.1	0.6	0.6	0.6	0.6
Phenylalanine	4.6	4.7	4.7	4.7	4.8
Threonine	4.7	4.8	4.8	4.8	4.9
Tryptophan	1.0	0.8	0.8	0.7	0.7
Tyrosine	3.1	2.8	2.8	2.6	2.3
Valine	5.9	6.5	6.5	6.0	5.7
Total EAA	51.2	49.2	49.1	47.7	48.8

[Adapted from Stilborn *et al*., (1997)]

2.4 Keratinolysis mechanisms: A hypothesis

Considerably, a numerous studies have been conducted on the keratin degrading proteolysis enzyme from various microorganisms. However, the keratinolysis or keratin decomposition mechanism is still debatable and unacquainted. At present, many researches are carried out to unravel the mystery of the decomposition of keratin.

2.4.1 Mechanical keratinolysis

Thoughtfully, the mechanical keratinolysis conjectures can only be applied by keratin decompose of filamentous fungi. Degradation of keratin occurrences explains the effect of fungal mycelial penetration on keratin. The elongation of fungal mycelial growth caused stress and enzymatic hydrolysis to the keratin substrate. The fungal invasion is necessary to help in exposing the reactive site for enzymatic action and is believed to produce exoproteases. However, another hypothesis assured the synergisms may occur in between mechanical and hydrolysis activities (Onifade *et al*., 1998). A greater understanding of the keratinolytic mechanism can be achieved with the help of technologically advanced microscope.

2.4.2 Sulfitolysis

The majority of researchers claimed that the reduction of disulphide bonds can cause keratin breakdown. This mechanism can be clearly explained as keratin consists of excessive amount of cystein that established the recalcitrant configuration of cross linkage in disulphide bridge. The decomposition of keratin started with the sequel of disulphite bond breakdown followed by degradation of keratinase enzyme. This process is known as sulfitolysis (Gupta and Ramnani, 2006). Several researchers have investigated the sulfitolysis occurrence in keratin degradation (Kunert, 1992). And they have reported that dermatophytic and non dermatophtic fungi used cystein as their sulphur and nitrogen source. Inorganic sulphur and other residues are released during cystein metabolisms process and the excess of sulphur are excreted back as sulphate and sulphite. At neutral or alkaline environment, sulphite released cystein and S-sulphocystein as elaborated in the equation below:

$$\underset{\text{(Cystein)}}{\text{Cys-S-S-Cys}} + \underset{\text{(Sulphite)}}{HSO_3^-} \longrightarrow \underset{\text{(Cystein)}}{\text{Cys-SH}} + \underset{\text{(S-sulphocystein)}}{\text{Cys-SSO}_3^-}$$

According to Kunert (1992), the similar reaction occurred in keratin. Degradation of keratin initiates by disulphide breakdown in accordance of sulphite act as a catalyst to sulfitolysis process. Subsequently, keratin degradation by enzyme hydrolysis took place (Malviya *et al*., 1992).

In point of fact, it is difficult to propose the sequence of reaction occurred in degradation of keratin. As for filamentous fungi and actinomycetes, the mycelium growth on the keratin substrate might initiate the mechanical keratinolysis followed by sulfitolisis in which the disulphide bonds are annihilated. Henceforth, keratinase enzyme will fully degrade the keratin. This mechanism is called proteolysis. This hypothesis is supported by most researchers including Wawrzkiewicz *et al*. (1991) and Mitola *et al*. (2002).

2.5 Diversity among keratinase producing microorganisms

Abundant chicken feather waste is accumulating in nature and creates an environmental issue because it takes slower decomposing time due to its rigid mechanical structure of the polypeptide (Brandelli, 2007). However, this bio-waste material can be degraded by the vast number of microorganisms including bacteria, fungi and actinomycetes (Yu *et al*., 1969; Asahi *et al*., 1985; Elmayergi and Smith, 1971; Abdel-Hafez and El-Sharoumy, 1990; Filipello-Marchisio, 2000; Mazotto *et al*., 2011). A lot of studies have been undertaken for as much prominence myriad of keratin degraded microorganisms shows an important role in the ecology and industry.

2.5.1 Fungi as keratinase producers

Moreover, there is always misinterpretation occurred in between keratinolysis and keratinophillic fungi. The difference between these two fungi is depending on the method used and the degree of keratin degradation (Sharma and Rajak, 2003). According to Filipello *et al*. (1994), keratinolysis fungi are a group of fungi which can completely degrade the keratin molecule and carried the similar characteristics as dermatophytic fungi and potentially pathogenic towards humans and animals. On the other hand, keratinophilic fungi capable to degrade either more simple substances associated with keratin or keratin degradation waste residue (Marchisio, 1986). Furthermore, most of the fungi that hydrolyzed keratin are from a class of dermatophytic fungi. They are frequently isolated from humans, animals and soil. However, keratinophilic fungi from dermatophyte group can cause mycosis to humans and animals (Marsella and Mercantini, 1986). This problem reduced the commercial value of keratinase derived although some studies considered have a biotechnological potential. Several non-dermatophytic fungi also produced keratinases and they include *Aspergillus oryzae* (Abdel-Rahman, 2001; Farag and Hassan, 2004; Bertsch and Coello, 2005; Ali *et al*., 2011). *Trichoderma atrvoviride* F6 (Cao *et al*., 2008), *Doratmyces microspores* (Gradisar *et al*., 2005), *Acremonium*, *Alternaria*, *Beauveria*, *Curvularia*, *Penicillium* (Marcondes *et al*., 2008) and *Myrothecium* (Moreira-Gasparin *et al*., 2009). Table 2.3 shows the diversity of fungi which can produce keratinase.

Table 2.3 Diversity among keratinase producing microorganisms from fungi

Microorganisms	References
Fungi	
Aspergillus fumigatus	Santos *et al*. (1996), Noronha *et al*. (2002)
Aspergillus oryzae	Abdel-Rahman, (2001), Farag and Hassan (2004), Bertsch and Coello (2005), Ali *et al*. (2011)
Aspergillus nidulans	Kaul and Sumbali (1999), El-Naghy *et al*. (1998)
Chrysosporium georgiae	Gradisar *et al*. (2005)
Doratmyces microsporus	Mignon *et al*. (1998)
Microsporum canis	Jindal *et al*. (1983)
Microsporum gypseum	Moreira-Gasparin *et al*. (2009)
Myrothecium verrucaria	Gradisar *et al*. (2005)
Paecilomyces marquandii	Anbu *et al*. (2005)
Scopulariopsis brevicaulis	Cao *et al*. (2008)
Trichoderma atrvoviride F6	Tsuboi *et al*. (1989), Siesenop and Bohm, (1995)
Trichophyton mentagrophytes	Qin *et al*. (1992)
Trichophyton schoenleinii	Singh (1997)
Trichophyton simii	Moallaei *et al*. (2007)
Trichophyton vanbreuseghemii	

In soil, keratinolysis fungi performed its biological function by degrading the keratinaceous materials like hair, feathers, nails, hooves and horn from dead animal bodies. Fungi are in the teleomorfisms or sexually stage in kleistotesium form. However, they are forming simple anamorfisms in their keratin host but if abundant source of keratin existed in the soil, they reproduced asexually and forming a lots of conidia. Ascotomata, the fruiting bodies are produced if depletion occurred in keratin source. In each ascotomata, there is ascus with eight ascospora. The ascospora will be in dormant phase and propagate as a new generation when keratin or nutrient source found back in the soil (Sharma and Rajak, 2003).

2.5.2 Bacteria as keratinase producers

Gram positive bacteria are well known to successfully produce keratinolytic activity, *Bacillus licheniformis* and *Bacillus subtilis* (Lin *et al*., 1999; Balaji *et al*., 2008); *Bacillus pumilus* (Kim et al., 2001; El-Refai *et al*., 2005) and *Bacillus cereus*, (Ghosh *et al*., 2008; Rodziewicz and Laba, 2008) were reported capable of disintegrating feathers and thus produced keratinase enzyme. However, Gram negative bacteria are also described as keratin degraders. Several strains reported to be able to produce keratinase such as *Xantomonas maltophila* (De Toni *et al*., 2002); *Vibrio sp.*kr2 (Sangali and Brandelli, 2000), *Alcaligenes faecali* and *Janthinobacterium lividum* (Lucas *et al*., 2003) and *Chryseobacterium sp.* kr6 (Riffel *et al*., 2007).

Researchers are also showing a great interest in thermofillic and alkaliphilic bacteria since keratin degradation facilitate in high temperature and pH in industrial process. For example *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996) and *Fervidobacterium islandicum* (Nam *et al*., 2002) were isolated from extreme environments whereas *Nocardiopsis* sp. TOA-1 were capable to produce keratinase in the strong alkaline environment (Mitsuiki *et al*., 2004). According to Friedrich and Antranikian (1996), *Fervidobacterium pennavorans* strain isolated from an Azores Island hot spring in Portugal can produce keratinase enzyme at 80ºC. Thermophillic bacteria can hydrolyzed rigorous keratin in high temperature due to its plasticity characteristic and caused its resistance to protease invasion (Suzuki *et al*., 2006).

Moreover, there are a few microorganisms exceeding the commercial value exploitation. Keratinase produced from *Bacillus licheniformis* and *Bacillus subtilis* have been studied further due to its effectiveness in the keratin degradation process (Manczinger *et al*., 2003 and Thys *et al*., 2004). Some of the enzyme produced give benefit to the medical field such as the keratinase from *Bacillus licheniformis* PWD-1 has been used in prion degradation in mad cow disease, Creuetfeldzt-Jacob disease, fatal familial insomnia, kuru and scrape (Shih, 1993). This discovery has given a rising hope to the suffered patients. Shih (1993) then manufactured Versazyme^{TM}, a commercial keratinase by using *Bacillus licheniformis* PWD-1 at Bioresource International Inc. Company. Table 2.4 shows a diversity of bacteria that able to produce keratinases.