ISOLATION AND CHARACTERIZATION OF MICROORGANISMS WITH KERATINOLYTIC ACTIVITY AND OPTIMIZATION OF KERATINASE PRODUCTION CONDITIONS

by

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF MICROORGANISMS WITH KERATINOLYTIC ACTIVITY AND OPTIMIZATION OF KERATINASE PRODUCTION CONDITIONS

Industrial enzymes gain increasing importance in several industries such as feed, fuel, food, pharmaceutics due to its advantages such as operation for bioprocesses at moderate conditions when compared to conventional chemical processes which typically occur at high temperature and pressure. Chicken feather can be considered as waste of the poultry farming. Increasing poultry farming practice renders the feather waste as a significant problem, not only for the environment but also for human health. On the other hand, feather has a high nutritious value due to high protein content from keratin and balanced amino acid profile. Conventional methods to process the feather waste, that is incineration or burying, loses the potential nutritional value of feather. The keratinase enzyme and the use of this enzyme to prepare so-called “feather meal” presents more environmental friendly and value added alternative to conventional feather waste management practice as this meal can immediately be used as an animal feed additive without losing its nutritional value.

This study focuses on isolation and characterization of a microorganism exhibiting keratinolytic activity. The operating conditions (pH, inoculum size, substrate concentration) are optimized for improved production and the produced enzyme is characterized on actual substrates. The enzyme exhibits higher keratinolytic activity when the microbial culture is induced with feather. The Michaelis Menten kinetic parameters for the produced keratinase enzyme are also determined and reported.

ÖZET

KERATİNOLİTİK AKTİVİTESİ OLAN MİKROORGANİZMALARIN İZOLASYONU, KARAKTERİZASYONU VE KERATİNAZ ENZİMİNİN ÜRETİM KOŞULLARININ OPTİMİZASYONU

Endüstriyel enzimler, besin, gıda, farmasötik, yakıt gibi alanlar sayesinde her sene etkinliğini arttıran bir teknoloji haline gelmiştir. Bunun başlıca sebebi geleneksel kimyasal proseserin yüksek sıcaklık ve basınçta işlem yapmasıdır. Kümes hayvancılığı, her sene üretimini artıran bir endüstri durumundadır. Dolayısıyla bu endüstrinin temel atıklarından olan tüy, sağlık ve atık olarak ciddi bir problem haline gelmektedir. Bu durumun yanısıra, tavuk tüyünün, protein içeriğinden dolayı, besin olarak katma değeri bulunmaktadır. Bu atıkların bertarafı yerine bu atıkları parçalayablecek enzimlerin kullanımı sayesinde, tüyün protein içeriği ve dengeli amino asit profilinden ötürü, hidrolizat hayvan yemi katkı maddesi olarak kullanılabilmektedir. Dolayısıyla, tüylerin enzimatik yollarla parçalanması, geleneksel yöntemler olan yakma ve toprağa gömme ile besin değerini kaybetmezken, bu yöntemlere kıyasla daha çevre dostu olmaktadır.

Bu çalışma, keratinolitik aktivite sergileyen mikroorganizmaların atık tüylerden izole edilmesi ve mikroorgnizmanın karakterizasyonunu amaçlamıştır. Ayrıca aktiviteyi arttırmak için üretim koşullarının (hücrelerin ortama aşılanma miktarları, substrat konsantrasyonunun ortama etkisi) iyileştirilme çalışmaları yapılmıştır. Bunların yanında enzimin karakteristik özelliklerinin tanımlanması üzerinde durmuştur. Mikroorganizmaların dışardan tüy ile indüklenmesi durumunda ortamda yüksek keratinolitik aktivite saptanmıştır. Ayrıca, üretilen enzimin Michaelis Menten kinetik parametreleri de hesaplanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

d	Day
g	Gram
h	Hour
k	Kilo, 10^3
L	Liter
M	Molarity: n/V, moles per volume
m	Meter
m	Milli, 10^{-3}
N	Normality: Mole concentration of H_3O^+ or (OH^-) per volume
n	Nano, 10^{-9}
α	Greek letter “alpha”
β	Greek letter “beta”
γ	Greek letter “gamma”
μ	Greek letter “mu”, Micro, 10^{-6}
4-pBPB	4-bromophenacyl bromide
AEBSF	4-(2-aminoethyl) benzenesulfonyl floride
BSA	Bovine Serine Albumin
Da	Dalton
DNS	Dinitrosalycylic
DTT	Dithiothreitol
dH_2O	Distilled water
ddH_2O	Double Distilled water
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EtOH	Ethanol
GC	Gas Chromatography
H_3O^+/H^+	Hydronium ion
HPLC	High Pressure Liquid Chromatography

IAA	Iodoacetamide
n.s.	Not Specified
OPA	o-Phthaldialdehyde
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
SSF	Solid State Fermentation
SmF	Submerged Fermentation
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TÜİK	Türkiye İstatistik Kurumu
wvol	Working Volume

1. INTRODUCTION

1.1. INDUSTRIAL ENZYMES

Catalysis on enzymes have become and still becoming more applied in industry due to several advantages such as catalyses of the reaction at harsh conditions like high temperature or pressure had been used for a long time in chemical industry which is highly costly or consumes too much energy. Furthmore, especially chemical catalyzers, such as sodium sulfide in dehairing process in leather industry [1], have a very negative impact on environment [2]. These are the main reasons that enzymes are more applied in industry.

Industrial enzymes are used in large quantities in pulp and paper, textile, detergent, dairy, baking, juice, brewing, animal feed, cosmetics and starch processing industries [3]. For example, in pulp and paper industry, amylase is used for degrading starch to reduce the viscosity; lipase for deinking and to control pitch in pulping; cellulase for improving texture and making fibers flexible; mannanase for increasing brightness by degrading glucomannan; laccase for brightness; β-xylanase for pulp bleaching. Similarly, textile industry uses amylase for desizing fabric; cellulase for making fabric smoother; pectinase for improving fiber extraction; laccase or glucose oxidase for bleaching. Detergent industry uses, protease for hydrolysing protein stains; lipase for hydrolyzing fatty stains; amylase for clearing starch residues; cellulase for increasing colour brightness and soften cotton. Dairy industry uses chymosin, lipase, lysozyme for producing cheese; β-galactosidase, lactase for breaking down lactose to avoid lactose intolerance problems. Baking industry, uses α-amylase for controlling bread volume; β-xylanase for improving dough handling and stability; oxidoreductase for increasing gluten strength; lipase for improving stability of the gas chambers in dough; protease for reducing protein in flour. Juice industry uses amylase and glucoamylase for hydrolysing starch and clarifying juice; pectinase for degrading cell wall pectins; cellulase and hemicellulase for lowering viscosity and maintenance of texture; laccase for increasing susceptibility of browning during storage; naringinase for controlling bitterness in citrus juices. Starch industry, uses α-amylase, pullulanase, neopullulanase, amylopullulanase, β-amylase, glucoamylase, isoamylase, glucose isomerase, glycosyl transferase enzymes are used for degrading various linkages in starch for desired structures.

Brewing industry, uses α-amylase for lowering viscosity by hydrolysing starch; β-glucanase for lowering viscosity by hydrolysing glucans into oligomers; pullulanase for securing the fermentability of the wort; amyloglucosidase for increasing glucose content; protease for increasing soluble protein and for malt improvement; pentonase and xylanase for hydrolysing pentosans of malt barley and wheat; α-acetolactate-decarboxylase (ALDC) for converting α-acetolactate to acetoin and avoiding formation of diacetyl. Animal Feed Industry, uses xylanase for degrading fibers in viscous diets; phytase for degrading phytic acid; protease for degrading protein, α-amylase for hydrolysing starch. Cosmetic industry, uses oxidase, peroxidase, polyphenol oxidase for hair dyeing; protein disulfide isomerase, glutathione sulfhydryl oxidase, transglutaminase for hair waving; papain, bromelain, subtilisin for making peeling effect on skin; amyloglucosidase, glucose oxidase are used in toothpastes and mouthwashes [3].

Industrially used enzymes are usually produced by bacteria or fungus. Typically, they are produced via either SmF or SSF. Commonly used funguses are *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus phoenicis*, *Cerena unicolor*, *Coriolus hirsutus*, *Lentinula edodus*, *Neurospora crassa*, *Penicilium citrinum*, *Penicilium capsulatum*, *Pencilium pinophilum*, *Pleurotus ostreatus*, *Spiecellum roseum*, *Sporotrichum pulverulentum*, *Trichoderma koningii*, *Trichoderma reesei*, *Trichoderma viride* [4]. Similarly, commonly used bacteria are *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Brevibacillus themoruber*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptomyces* sp., *Streptomyces rectus*, *Thermus aquaticus*, *Vibrio proteolyticus* [3,5,6].

The enzymes are produced using either highly producing natural isolates or using industrial workhorse microbes equipped additionally with DNA encoding the enzyme of interest, using recombinant DNA technology. Isolation of microorganism producing enzyme of interest, the “wild-type route”, is the old, common, relatively inexpensive method; yet the success rate of finding a good producer depends heavily on screening throughput. The alternative, so-called the “recombinant route” exploits the developments in recombinant DNA technology to generate strong enzyme producers that are easy-to-grow and have specific traits for separation and purification processes. After production, separation and purification

techniques are performed in different levels for the final product. Typical workflow of enzyme production is depicted in **Figure 1.1**.

Currently, major companies producing industrial enzymes are generally from Europe (Denmark, Netherlands, Switzerland etc.), USA, and Japan. These companies can be listed as, Novozyme which produces enzymes for food, beverage, bioenergy, biopharmaceutics, and technical enzymes; Dupont (Genencor/Danisco) for biofuel, textile, food, detergents; DSM for animal nutrition, personal care, pharmaceutics; Roche for diagnostics, pharmaceutics; Amano for pharmaceutics, food processing, biotransformation; AB Enzymes for food, textile, detergent, pulp and pare, biofuel; BASF for feed additives, pharmaceutics, detergent; Chr. Hansen produces enzymes for cheese; Shin-Nihon for animal nutrition, food, biofuel. ADM, KAO, Biozyme, Verenium, Iogen, Dyadic, Meiji, Enmex, Niagase are some other companies that have good market share which produces enzymes for similar industries [3].

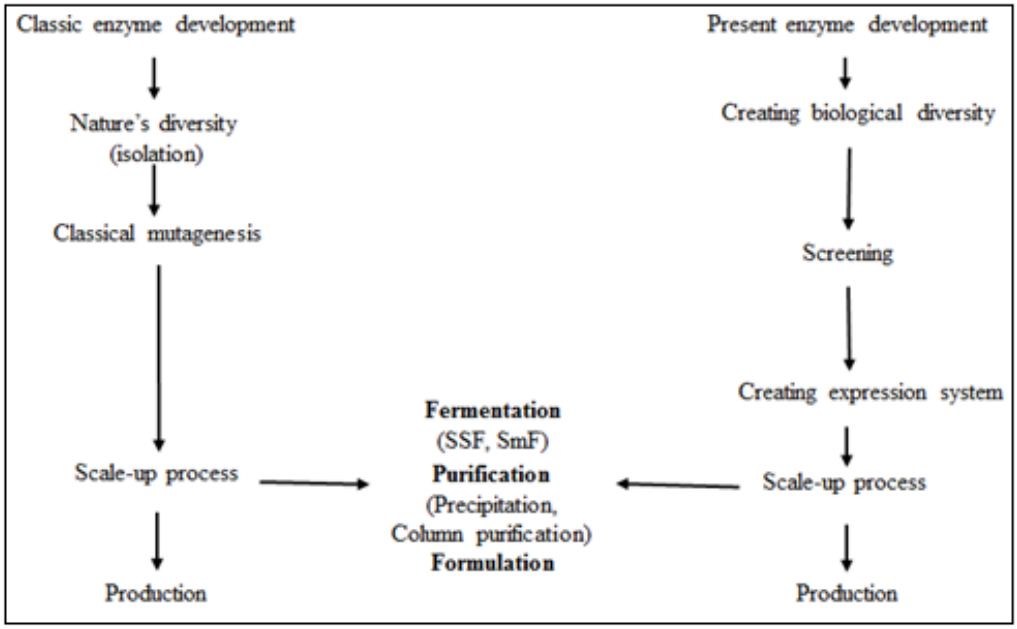

Figure 1.1. Enzyme production diagram.
The figure is adapted from Kirk et al., 2002 [7]

Enzyme demands have been affected economies very well such as in USA, Canada, Europe, Japan which are now relatively stable, while Asia, Middle East, and Eastern Europe currently have very good growing. The highest growth rate is in leather market following by

bioethanol, beverage, and food industries [8]. The global market for industrial enzymes in 2012 was $4.5 billion, in 2013 $4.8 billion, and in 2018 it is expected to be $7.1 billion. In 2013; food and beverages was valued $1.3 billion, detergent enzyme market was valued about $1.1 billion. In 2018; food and beverages is expected to be valued $1.7 billion and detergent enzymes at $1.8 billion [9].

Among the industrial enzymes, proteases have the most of the share among the other type of enzymes with approximately 40% in 2002 with a expectation of increasing further years [5]. Detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery are the application areas that this proteases are used.

Proteases are essential enzymes in every organism such as prokaryotes, fungi, plants, and animals. Microbial proteases are classified due to active sides which they show activity under alkaline, acidic, and neutral conditions. These groups are: serine- (EC.3.4.21), cysteine- or sulphydryl- (EC.3.4.22), aspartic- (EC.3.4.23), and metallo- (EC.3.4.24) type proteases [5]. Microbial keratinases are alkaline proteases which have both metallo and serine types.

There are many companies that produces commercial proteases. Novo Nordisk produces, Alcalase from *Bacillus licheniformis* for detergent industry, silk degumming processes; Savinase from *Bacillus* sp. detergent, textile industries; Esperase from *Bacillus lentus* for detergent, food, and silk degumming; Biofeed pro from *Bacillus licheniformis* for feed industry; Durazym from *Bacillus* sp. for detergent industry; Novozyme 243 from *Bacillus licheniformis* for denture cleaners; Nue from *Bacillus* sp. for leather. Genencor produces, Purafact from *Bacillus lentus* for detergent; Primatan for leather industry. Amano produces, Collagenase from *Clostridium* sp. for technical uses; Amano protease S from *Bacillus* sp. for food industry. Nagase produces, Bioprase from *Bacillus subtilis* is used for cosmetic, pharmaceutics, and detergent industries; Ps. Elastase from *Pseudomonas aeruginosa* is used for researches [5].

1.2. OBTAINING VALUE ADDED PRODUCTS FROM POULTRY FARM WASTES

Poultry farming increases every year due to nutritional needs increasing human population (**Figure 1.2**). Although between 2005-2008 there were serious avian flu in Turkey that the number of animals in the poultry farming significantly decreased, overall production increased. Feather is an abundant waste in poultry industry, assuming 75 g feather/fowl, summing up to 20×10^6 kg of feather in 2015, in Turkey.

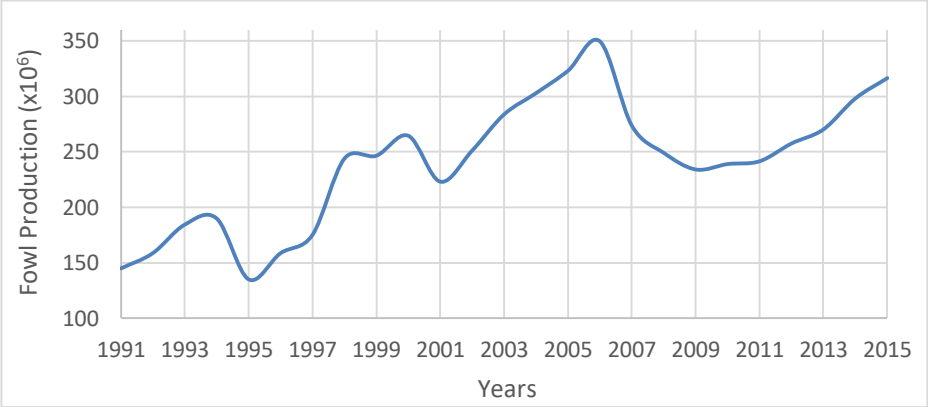

Figure 1.2. Fowl Production between 1991-2015 in Turkey (TÜİK).

Current methods used in feather waste management are incineration or burying. These methods are energy consuming and do not exploit enough the potential nutritional value of feathers. Alternatively, enzymatic degradation of feather offers cleaner and environmentally friendly solutions to this. The resulting enzyme, keratinase, will degrade feather waste and can further be used in different areas such as agriculture, cosmetics, detergent, pharmaceutical industries.

Feather, contains keratin by 90% in weight [10]. Feather has different parts presented in **Figure 1.3** and the amino acid composition of each part is different. The amino acid composition of the barb is presented in **Table 1.1**.

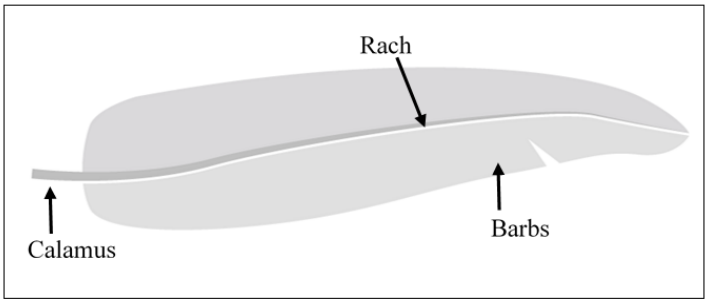

Figure 1.3. The morphology of feather.

Table 1.1. Amino acid composition of whole feather barbs (μmoles/g). The table was adapted from Harrap et al., 1962 [11].

Amino Acid	**μmoles/g**	**Amino Acid**	**μmoles/g**
Alanine	446	Lysine	71
Arginine	384	Methionine	15
Aspartic Acid	519	Phenylalanine	296
Cysteine	768	Proline	1056
Glutamic Acid	686	Serine	1236
Glycine	970	Threonine	437
Histidine	17	Tryptophan	22
Isoleucine	383	Tyrosine	118
Leucine	605	Valine	705

1.3. KERATIN

Keratin is structural protein that exists in many organisms. In nature it is used for protecting or covering such as in skin, wool, fur, baleen, hair, spines, quills, and in protective armours; for directly defences or aggressions horns, claws, nails, beaks, teeth, and slimes; for motions it is used in hooves and feathers [12].

Keratin is fibrous protein that is insoluble in water and it is resistant to many enzymatic activities. In α-keratins, high number of dissulfide bonds are the reason for this. Keratinous structures are majorly configured in two types: α-helix and β-sheet. This two configuration

type makes keratins in divisions such as α-keratin, β-keratins, γ-keratins [13], and mixed keratins which contains both α-keratin and β-keratins.

α-keratins are based on α-helices. Molecular mass of α-keratins are between 40-68 kDA [14]. Stratum corneum, wool, hair, quills, horns, hooves, nails, baleen, and slime are in α-keratin group [15].

β-keratins are based on β-pleated sheets. Molecular mass of β-keratins are between 10-22 kDa [14]. Feathers, beaks, claws, scales are in β-keratin group [15].

Feather are used by vertebrates for flight, camouflage, insulation. Chicken feather's structure contain both α-keratin and β-keratin groups in different parts. Barbs are α-keratins while raches contains high amount β-sheets [16]. α-keratins has low sulphur content comparing to β-keratins. So sulphur content also differ in between feather parts.

Amorphous keratins or γ-keratins is a part of matrix. It is rich in cysteine. γ-keratins' molecular mass is around 15 kDa [13].

Keratin has a high protein content. Before enzymatic degradation feathers were baked in high temperature and high pressure for degradation to be used as feather meal. This procedure, besides being expensive, have made loss of essential amino acids like methionine, lysine, and tryptophan. That shows the necessity of enzymatic degradation for making feather meals.

1.4. KERATINASE

Keratinases or keratinolytic proteases are enzymes that degrades keratin containing substrates. Most of the keratinases are serine proteases but some of them are metallo proteases [17]. Predominantly this enzyme is extracellular but also cell bound [18,19] and intracellular [18,20] types have been reported. It is produced by bacteria and fungi. Some of keratinolytic fungal sources are *Alternaria alternata, Aspergillus candidus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus parasiticus, Aspergillus terreus, Botryotrichum piluliferum, Chrysosporium carmichaeli, Chrysosporium indicum, Chrysosporium keratinophilum, Chrysosporium queenslandicum, Chrysosporium tropicum, Cladosporium cladosporioides, Fusarium oxysporum, Fusarium solani, Microsporum*

audouinii, *Microsporum canis*, *Microsporum cookei*, *Microsporum gypseum*, *Myceliophthora* sp., *Myrothecium verrucaria*, *Paecilomyces variotii*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Penicillium glabrum*, *Scopulariopsis brevicaulis*, *Scopulariopsis brumptii*, *Stachybotrys chartarum*, *Trichoderma viride*, *Trichophyton ajelloi*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton terrestre* as listed in Blyskal, 2009 [21]. Bacterial sources are listed in **Table 1.2** where production conditions and in **Table 1.3** where characteristics of keratinase's are given.

Sources of enzymes ,that are studied, are generally isolated from poultry waste, soil, lake, hot springs [10]. Beside typical keratinous substrates such as wool and feather, there are other substrates studied for inducing microorganism and producing enzymes that have keratinolytic activity. These are casein, silk, skim milk [22], fish silage, shrimp waste, collagen [23]. For activity measurement of keratinase enzyme; azokeratin, keratin azure, hair, feather, keratin powder [24] are used as substrate as it is shown in **Table 1.4**.

1.4.1. Isolation, Production Of Keratinolytic Microorganisms, Characteristics Of Keratinase And Measurements Of Keratinase Activity

Production of keratinase is typically studied in SmF, several examples are given in **Table 1.2**. But there are also some studies that SSF is used which is fungi used [25-27].

Molecular weight of keratinase differs from 18 to 240 kDa [28]. Optimum pH range for activities for enzyme have been reported between 6-12.5 which shows that it is an alkaline protease. There are some keratinases that has optimal pH in acidic conditions [28]. They are mostly fungal keratinases. Optimum temperature working range for activity have been reported to be between 40-100°C. Normally maximum optimum temperature is around 75°C and over this temperature the reason is extremophiles [19,28, 29].

Table 1.2. Enzyme production conditions.

Microorganism	Source	Substrates (g/L)	Production Conditions	Specific Activity (Maximum observed)	Reference
Bacillus licheniformis ER-15	Soil	Feather (5), soy flour (30), glucose (4)	pH 7.0, temperature 37°C, 48 h, 250 rpm	514.5±4.38 (U/mL)	[30]
Bacillus licheniformis PWD1	Poultry waste	Hammer milled chicken feather (10)	pH 7.5, temperature 50°C, 30 h, 120 rpm	-	[31]
Bacillus subtilis KD-1	Mutant	feathers (10)	pH 7.5, temperature 23°C, 30 h, 200 rpm	83.6±2.11 (U/mL)	[22]
Bacillus pumilis A1	Slaughter house	Feather meal (30), soy peptone (3.15)	pH 6, temperature 30°C, 28 h, 250 rpm	87.75 (U/mL)	[32]
Bacillus megaterium F7-1	Poultry waste	Feather (16), skim milk (6)	pH 6.5-11, temperature 25-35°C, 5 d, 200 rpm	-	[33]
Brevibacillus sp. AS-S10-II	Soil	Casein (14)	pH 13.3, temperature 50°C, 48 h, 200 rpm	$923x10^3$ (U/L)	[34]

Table 1.2. Continued

Microorganism	Source	Substrates (g/L)	Production Conditions	Activity (Maximum observed)	Reference
Chryseobacterium sp. strain kr6	Feather waste	Feather (30)	pH 9, temperature 23°C, 16 h, 125 rpm	1,559 (U/mL)	[35]
Fervidobacterium islandicum AW-1	Geothermal hot spring	Feather (8)	pH 7.0, temperature 70 °C, 48 h,	-	[19]
Kocuria rosea LPB-3	Soil	Milled feathers (30), yeast extract (0.1)	pH 7.5, temperature 40 °C, 14 h, 400 rpm, 1.8vvm	-	[23]
Meiothermus sp. strain I40	Hot Springs	Feather (0.8), yeast extract (2.7), tryptone (1.6)	pH 7.0, temperature 51.7°C, 72 h, 120 rpm	-	[36]
Microbacterium sp. strain kr10	Feather waste	Feather (12.5)	pH 7.0, temperature 25°C, 4 d, 125 rpm	202.7 (U/mL)	[37]

Table 1.2. Continued

Microorganism	Source	Substrates (g/L)	Production Conditions	Activity (Maximum observed)	Reference
Paracoccus sp. WJ-98	Soil of poultry factory	Feather (10), urea (0.5)	pH 7.5, temperature 37°C, 84 h	90 (U/L)	[38]
Pseudomonas sp. 3096-4	Decomposing wool	Raw wool (10)	pH 7.6, temperature 30°C, 48 h, 160 rpm	-	[39]
Stenotrophomonas maltophilia R13	Rhizospheric soil of reed	Feather (2), glucose (1), polypeptone (1.2)	pH 7.0, temperature 30°C, 48 h, 200 rpm	82.3±1.0 (U/mL)	[40]
Streptomyces sp. MS-2	Marine sediment	Whole feather (10)	pH 8.0, temperature 25°C, 72 h, 150 rpm	-	[41]
Thermoanaerobacter keratinophilus	Geothermal hot spring	Feather (5)	pH 6.8, temperature 70°C, 10 d	-	[29]
Xanthomonas sp. P5	Rhizospheric soil	Feather (1.4), gelatine (1), fructose (3)	pH 8.0, temperature 30°C, 5 d, 200 rpm	69.0±0.6 (U/mL)	[42]

Table 1.3. Enzyme activity parameters.

Microorgnism	Molecular Mass	Activity (Maximum observed)	pH (Optimum)	Temperature (Optimum)	Activator	Inhibitor	Reference
Bacillus licheniformis PWD-1.	33 kDa	-	7.5	50°C	-	-	[43]
Bacillus cereus DCUW	80 kDa	-	8.5	50°C	-	PMSF, Pepstatin, E-64, EDTA	[44]
Bacillus pumilis A1	-	87.75 (U/mL)	8.5	55°C	Ca^{2+}, Na^{+}, β-Mercaptoethanol	Mn^{2+}, Cu^{2+}, Hg^{2+} PMSF, EDTA	[32]
Brevibacillus sp. strain AS-S10-II	83.2 kDa	$923x10^{3}$ (U/L)	12.5-13	45°C	SDS, Tween 20, Urea	PMSF, 4-pBPB, IAA, EDTA, SDS, DTT	[34]
Clostridium sporogenes	28.7 kDa	632 (U/mg)	8	55°C	-	-	[45]
Chryseobacterium sp. kr6	64 kDa	967 (U/mg)	8.5	50°C	Ca^{2+}, Mg^{2+}, Cd^{2+}	EDTA, EGTA, 1,10-Phenanthroline, PMSF, IAA, 2-Mercaptoethanol, DTT, SDS	[46]

Table 1.3. Continued

Microorgnism	Molecular Mass	Activity (Maximum observed)	pH (Optimum)	Temperature (Optimum)	Activator	Inhibitor	Reference
Fervidobacterium islandicum AW-1	97 and >200 kDa*	-	9	100°C	n-Octyl-β-D-glucopyranoside,	Triton X-100, SDS, Cholic acid, PMSF	[19]
Kocuria rosea	240 kDa	-	10	40°C	-	AEBSF, chymostatin, antipain	[47]
Stenotrophomonas maltophilia	35.2	-	7.8	40°C	-	PMSF, $PbCl_2$, $CdCO_3$, $HgCl_2$	[48]
Streptomyces pactum DSM 40530	30 kDa	-	7-10	40-75°C	-	SDS, Triton X-100, Thioglycolate, PMSF, EDTA	[49]
Thermoanaerobacter keratinophilus	135 kDa	-	7 (60°C) 8 (85°C)	60°C (pH 7) 85°C (pH 8)	-	PMSF, Pefabloc SC, IAA, Pepstatin, EDTA	[29]
Xanthomonas maltophilia	36 kDa	18461 (U/mg)	9	60°C	-	PMSF, EDTA	[50]

Table 1.4. Keratinase activity measurement conditions for comparison.

Unit Definition of Keratinolytic Activity	Substrate	Reference
0.01 absorbance increase at 450 nm from 15min 50°C incubation is equal to 1U keratinase activity	Azokeratin	[43]
1 absorbance increase from azo dye at 595 nm from 3 h 42°C incubation is equal to 1U keratinase activity	Keratin Azure	[51]
0.1 absorbance increase at 595 nm from 1h 50°C incubation is equal to 1U keratinase activity	Keratin Azure	[52]
0.1 absorbance increase at 280 nm from 30 min 37°C is equal to 1U keratinase activity	Keratin Powder	[53]
0.01 absorbance increase at 660 nm from 5h 30°C incubation is equal to 1U keratinase activity	Keratin Powder	[54]
0.01 absorbance increase at 280 nm from 1 h 60°C incubation is equal to 1U keratinase activity	Feather	[55]

1.4.2. Applications Of Keratinase Enzyme

Keratin has a high (up to 90%) protein content. Before enzymatic degradation feathers were baked in high temperature and pressure for degradation to be used in meal. This procedure, besides being expensive, have made loss of essential amino acids like methionine, lysine, and tryptophan. That shows the necessity of enzymatic degradation for making feather meals. Keratinases have various applications areas such as in feeds, fertilizers, detergent industry, leather industry, prion degradation, cosmetics, pharmaceutics.

Beside using it in animal feed industry, it can also be used as fertilizers for plants. The high protein content could improve directly plant growth and stimulate microbial activity in soil and help growth of plant indirectly.

In detergent industry keratinases are used for cleaning substrates that are not cleaned by most proteases sufficiently. Also it is used to open drains clogged with keratinous wastes.

One of the areas that this enzymes is used is leather industry. Leather industry is one of the important causes for environmental pollution because of usage of chemicals in leather processing. Biocatalytic leather processing include proteases, lipases and some other enzymes. Using keratinases could help in dehairing process where proteases are not fully effective. Using keratinase along with other biocatalysts could prevent using these toxic chemicals and make this industry more environmental friendly.

Prions are protein materials that causes viral-like diseases. There are reports that microbial keratinases could be used for prion decontamination. This is important for decontaminating surgical instruments, contact lenses, or feather meals that are going to use feed additives [24,56].

Commercial keratinases (or enzymes which are used for their keratinolytic activities) that are used in this topics, could be listed as; Protease P4860 that is produced from *Bacillus licheniformis* by Novozymes, Protease P3111 from *Bacillus* sp. by Novozymes, Versazyme from *Bacillus licheniforms* by Bioresource International, Valkerase from *Bacillus licheniformis* PWD-1 by BioResource, Prionzyme from *Bacillus licheniformis* by Genencor, Proteinase K from *Tritirachium album* by New England Biolabs, PURE100 by *Bacillus licheniformis* PWD-1 by Zurko Bioresearch. Except Prionzyme and Proteinase K, they are specifically produced for feather or keratin containing materials but these two enzymes are produced only for prion degradation [57,56].

1.5. THE AIM OF THE STUDY

The main aim of this study is to isolate and characterize a microorganism that has high keratinolytic activity. Following this, producing the extracellular keratinase enzyme from isolated microorganism that is industrially important which will be used in animal feed additive industry.

For this purposes, characterizing the microorganism with observing growth kinetics, fermentation conditions and then to optimize conditions for higher enzyme activities is important. Also characterization of the enzyme is the next step to observe. Purification of enzyme is last step to perform, for observing if purified enzyme shows acceptable activity for industrial usage.

2. MATERIALS

2.1. MEDIA AND CHEMICALS

Chemical	Company
Acetic acid	Merck
Ammonium persulfate (APS)	Sigma Aldrich
Ammonium sulfate	Sigma Aldrich
Bovine Serine Albumin (BSA)	Sigma Aldrich
Coomassie Brilliant Blue	Sigma Aldrich
DTT	Sigma Aldrich
EDTA	Sigma Aldrich
Ethanol (EtOH)	Sigma Aldrich
Hydrochloric acid	Sigma Aldrich
Glycerol	Sigma Aldrich
Glycine	Sigma Aldrich
Keratin Azure	Sigma Aldrich
Magnessium sulfate heptahydrate	Sigma Aldrich
Pageruler Prestained Protein Marker (10-170 kDa)	Thermo Scientific
Potassium phosphate dibasic trihydrate	Sigma Aldrich
Potassium phosphate monobasic	Sigma Aldrich
Sodium chloride	Merck
Sodium hydroxide	Sigma Aldrich
Sodium dodecyl sulfate	Sigma Aldrich
Protease from *Bacillus* sp.	Sigma Aldrich
Trizma® base	Sigma Aldrich
Tryptic Soy Agar (TSA)	Lab M
Tryptic Soy Broth (TSB)	HiMedia

2.2. LABORATORY EQUIPMENTS AND DEVICES

Equipment	**Company**
96 well plate	Isolab
Bottles (100mL, 250mL, 500mL, 1000mL)	Isolab
Cryo tubes	Isolab
Erlenmeyer Flasks (250mL, 500mL, 1L, 2L)	Isolab
Eppendorf tubes (1.5mL, 2mL)	Expell
Falcon tubes (15mL, 50mL)	Axygen
Graduated cylinders (50mL, 250mL, 500mL)	Isolab
Inoculation loops	Isolab
Micropipettes (1-10µL, 10-100µL, 20-200µL, 100-1000µL)	Eppendorf
Micropipette tips (10µL, 200µL, 1000µL)	CAPP
Petri Dishes (120x17mm, 60x15mm)	Isolab
Serological pipettes (5mL, 10mL, 25mL)	Isolab
Suction bulbs	Isolab

Device	**Company**
Autoclave	Hirayama HV-85
Autoclave	Tuttnauer 5050 ELV
Balance	Shimadzu AUW220D
Balance	Radwag PS 4500/C/1
Balance	Desis
Biological Safety Cabin-Class II, Type A2	HFsafe-1200
Biological Safety Cabin-Class II, Type A2	Labculture®-ESCO
Biological Safety Cabin-Class II	SafeFAST Elite
Centrifuge	Eppendorf^{TM} 5424
Centrifuge	Beckman Coulter Avanti® J-E
HPLC	Shimadzu
Fridge	Arçelik
Fridge	Sanyo
Incubator	Memmert
Incubator	Binder
Incubator Shaker	Sartorius Stedim CERTOMAT® IS
Incubator Shaker	New Brunswick Innova® 44
Magnetic Stirrer	Benchmark
pH meter	Mettler Toledo
Vortex	Scilogex MX-S
Water bath	Grant SUB Aqua 12 Plus
Water source	arium®pro Sartorius
Water source	arium®advance Sartorius

3. METHODS

3.1. MICROBIOLOGICAL METHODS

3.1.1. Isolation Of Keratinolytic Microrganisms

Isolations are performed as described in Cai et al., 2008 [22]. Agar plates are prepared by using g/L: NaCl (0.5), KH_2PO_4 (1.4), K_2HPO_4 (0.7), $MgSO_4$ (0.1), chicken feather (10), and agar (10). After preparation of media, it is autoclaved at 121°C for 15 minutes. After autoclaving agar is poured to petri dishes.

Saline solution is prepared by using 8.5-9 g/L NaCl. 10 g/L of feather is added in saline solution and it is incubated for different incubation times, which is 30 minutes and 1 week, at 37°C. 300 µL samples are taken from solution and spreaded to feather containing agar dishes and placed for incubation in 37°C for two days. After two days, samples have grown on agar. Isolates, which had incubation time of 30 minutes in saline solution, numbered between 29-44. Isolates, which had incubation time for 1 week in saline solution, numbered between 61-70.

3.1.2. Selection Of Keratinolytic Microorganisms For Their Keratinolytic And Proteolytic Activites

Isolated microorganisms numbered between 29-44 and between 61-70 were tested for their proteolytic activity on skim milk agar. These isolated microorganisms are grown for 16-18h at 37°C, 150 rpm on TSB. Then, microorganisms are inoculated to inducing media which have either feather or soy bean. Inducing media contains g/L: NaCl (0.5), KH_2PO_4 (1.4), K_2HPO_4 (0.7), $MgSO_4$ (0.1), chicken feather or soy bean (10). After preparation media is autoclaved at 121°C for 15 minutes. Inducing-media are inoculated with isolated microorganism and incubated for 3 days 37°C, 150 rpm.

Skim milk agar for screening proteolytic activity is performed with modifications on Alnahdi, 2012 [58]. 100g/L skim milk and 20g/L agar prepared separately and mixed 1:1 by

volume. After mixing skim milk agar is autoclaved at 121°C for 5 minutes. Agar is poured to 60x15 mm petri dishes. After agar is solidified, a hole of 1 cm diameter is made in the middle of agar for pouring the induced media.

40 µL samples are taken from induced-isolates and poured to skim milk agar plates. Agar plates have been incubated at 37°C for 3d. Each day clear zones are observed. Different inducers (feather and soy bean) have been used separately.

3.2. ENZYME PRODUCTION

Culture medium is prepared by using g/L: NaCl (0.5), KH_2PO_4 (1.4), K_2HPO_4 (0.7), $MgSO_4$ (0.1), chicken feather (10) [22]. After preparation medium is autoclaved at 121°C for 15 minutes. Isolates are grown for 16-18 h at 37°C 150 rpm in TSB and inoculated to the feather containing medium.

3.3. ENZYME SEPERATION AND PURIFICATION

Ammonium sulfate is added to supernatant while slowly stirring at 4°C. For certain percent saturation, the amount that is needed to be added per volume is given in **Figure 3.1**. After addition it is centrifuged at 10,000 g for 10 minutes. Pellet is dissolved in Tris-HCl buffer. Molarity of buffer can change between 20-100 mM as done in literature. Also pH of the buffer is around 8 in literature [50,59-61]. After that, solution is added inside MEMBRA-CEL® md77 14x100 CLR membrane and put in a surrounding solution, that is the same buffer which pellet is dissolved, for overnight at 4°C.

Initial concentration of ammonium sulfate - % saturation at 0°C	Final concentration of ammonium sulfate - % saturation at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	gram of ammonium sulfate to add 1L solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Figure 3.1. Ammonium sulfate precipitation table

3.4. ANALYTICAL METHODS

3.4.1. Enzyme Characterization

3.4.1.1. Molecular Weight Determination

The molecular weight of enzyme's is estimated by SDS-PAGE [62]. 12% (w/v) polyacrylamide resolving gel and 5% (w/v) polyacrylamide stocking gels are used. Coomassie Brilliant Blue R-250 is used for staining proteins. The obtained bands on SDS page is used to determine the molecular weight of the protein, which is estimated with Pageruler Prestained Protein Marker (Thermo Scientific).

3.4.1.2. Enzyme Activity Determination and Kinetic Calculations

0.2 mL enzyme + 0.8 mL 10 mM Tris-HCl pH 8.5 with Keratin Azure (as a substrate) is used. The mixture is shaken at 200 rpm for 1 h at 50°C. It is centrifuged at 15,000 rpm for 10 minutes. Activity is read from the blue dye that is released to the solution. One unit of keratinolytic activity (U) is calculated by increase of 0.1 absorbance at 595 nm in one hour [52]. Blank is every enzyme's own replicates which is boiled (96-100°C) for 10-15 minutes.

Kinetic parameters of activity (K_m, V^{max}) is calculated with Michaelis-Menten equation which is shown in **Equation 3.1**.

$$\nu = \frac{d\,[P]}{dt} = \frac{V_{max} \times [S]}{K_m + [S]} \tag{3.1}$$

3.4.2. Microbial Identification System (MIS) With Fatty Acid Methyl Ester (FAME) Analysis

Fatty acid methyl ester analysis is a method to track microorganisms since every microorganisms has its fingerprint of fatty acid methyl esters. FAME analysis has five steps.

Harvesting, saponification, methylation, extraction, and washing. Isolated microorganisms were grown at 37°C on TSA for 1 day.

First, cells were harvested from agar plate with the help of loop and were put in tube which contains 1 mL of Reagent 1. After it is vortexed for 5-10 seconds, tube is incubated at 100°C for 5 minutes. Then again it is vortexed for 5-10 seconds and is cooled at room temperature for 25 minutes.

Then for methylation 2 mL of Reagent 2 is added to tube and vortexed for 5-10 seconds. It is ıncubated in 80°C for 10 minutes.

After rapidly cooled, 1.25 mL Reagent 3 is added. Sample is mixed for 10 minutes laboratory rotator. Bottom phase is removed with pasteur pipette.

The top phase is washed with 3.0 mL Reagent 4. After mixed for 5 minutes tubes are rotated and the top phase is removed for analysis and transferred to GC vials.

Microorganism is identified by gas chromatography (GC) followed by FID detector from FAME profiles by TSB6 database with Microbial Identification System version 6.0

Reagent 1: 45g sodium hydroxide (certified as ACS) dissolved in mixed 150mL methanol (HPLC grade):150mL deionized distilled water. Reagent 2: 325mL 6N HCl mixed with 275mL methanol (HPLC grade). Reagent 3: 200 mL hexane (HPLC grade) mixed with 200mL Methyl tert-butyl ether (HPLC Grade). Reagent 4: 10.8 g sodium hydroxide (certified ACS) is dissolved in 900 mL deionized distilled water.

3.4.3. Cell Optical Density (OD) Measurements

Cell OD is measured as described: 1 mL of sample is taken to cuvettes and measured using spectrophotometer at wavelength of 540 nm. Broth without any cells is used as blank.

3.4.4. Total Protein Determination

Total protein determination is performed with Bradford method, modified with volume [63]. 5µL of samples and standards are transferred to 96 well plate. 250µL of Bradford reagent is

added and mixed with samples and standards. After 5 minutes at room temperature the readings are done in spectrophotometer at 595 nm. All pipetting and transferring the plate to spectrophotometer are performed in dark and standard curve is prepared with BSA.

3.4.5. Reducing Sugar Determination

Reducing sugar determination is performed using modified from DNS method described before [64,65]. DNS reagent is prepared by mixing 1g 3,5dinitrosalicylic acid, 30 g potassium sodium tartarate and 20 mL 2N NaOH in final volume of 100 mL which is completed with dH_2O.

0.5 mL samples or standards are mixed with 0.5 mL DNS reagent and incubated at 96-100°C for 10 minutes. Readings are done at 540 nm in spectrophotometer.

3.4.6. Amino Acid Determination Via High Pressure Liquid Chromatography (HPLC)

Amino acid determination is performed as mentioned in Application Note of Knauer [66] which method is given in **Table 3.1** and procedure is given in **Figure 3.2**. In this procedure amino acids have to been derivatized with OPA for pre-column derivatization. Borate buffer's concentration is 0.5 M and pH is 9.2. OPA reagent contains 10mg o-phthaldialdehyde dissolved in 9mL methanol, 1 mL borate buffer, and 100µl mercaptoethanol. Neutralization buffer contains, 100 mL 0.05 M sodium acetate added with 25 mL methanol, pH is brought to 6.1.

Table 3.1. Method of HPLC for amino acid measurements.

Column	XBridge C18		
Eluent A	50 mM Sodium acetate pH 7.2		
Eluent B	50 mM Sodium acetate pH 7.2/Methanol 25:75 (v/v)		
Gradient	Time (min)	%A	%B
	0.00	90	10
	1.50	90	10
	5.00	50	50
	8.00	0	100
	10.00	0	100
	13.00	90	10
Flow Rate	0.9 mL/min		
Injection volume	5 µL		
Run Time	13.0 min		
Column temperature	45 °C		
Detection	UV, 230 nm		

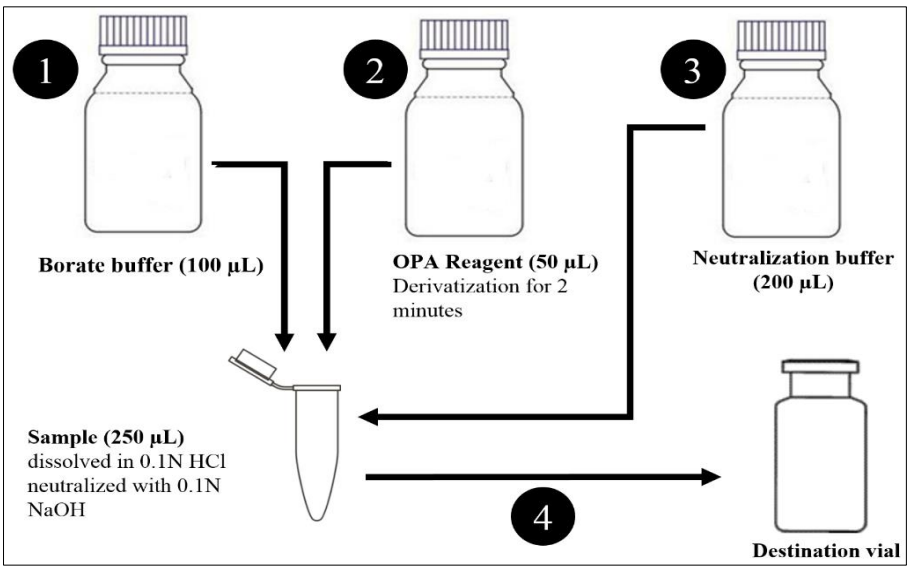

Figure 3.2. OPA derivatization procedure

4. RESULTS

4.1. CHARACTERIZATION OF MICROORGANISM

4.1.1. Skim Milk Agar Clear Zones

The feather waste has been used to isolate microorganisms using a saline solution, the colonies are then transferred to non-selective TSB medium then the enriched culture has been used as inoculum to the feather containing medium. After 3 days of incubation on feather medium, 40 µL samples are collected and tested for proteolytic activity using clear zone assays on skim milk agar (**Figure 4.1**)

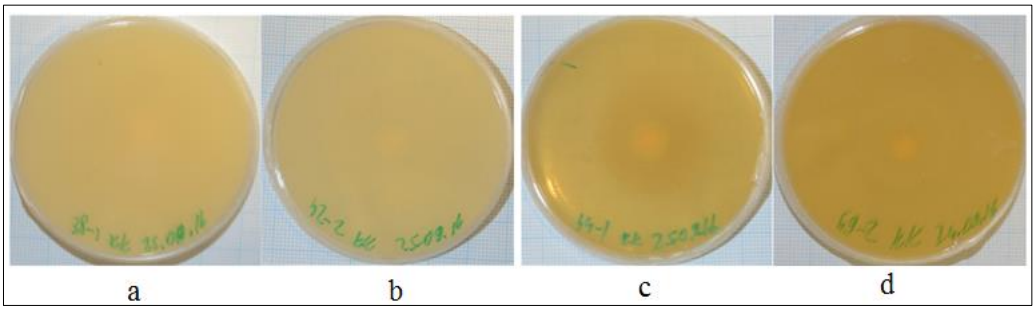

Figure 4.1. Some examples of isolations numbered between 29-44 and 61-70. They were induced with feather in liquid culture. Clear zones were observed

Four samples are selected among the isolates that degraded skim milk and made clear zones. These were isolates number 39, 44, 67 and 69. Isolates numbered between 29-44 were incubated 30 minutes at 37°C in saline solution while isolates numbered between 61-70 were incubated 1 week at 37°C. Comparing both groups, the latter (with isolates numbered 61-70) degraded skim milk agar more effectively so this group contained microorganisms with higher keratinolytic activity.

After selecting isolates, the selected microorganisms have been enriched with soybean and feather. This is followed again by the skim milk agar clear zone experiment, the results of which is presented in **Figure 4.2** and **Figure 4.3** as clear zones from microorganisms and

their broth's supernatant's. The isolate numbered 69 is selected among them for further studies because it showed better activity (with both inducers for proteolytic activity) than others. Here, the selection was not only based on the area of clear zone but also, the time that the organism required to clear (fast the better) as well as the clarity of the cleared zone (clearer the better; some isolates yielded "misty" clear zones)

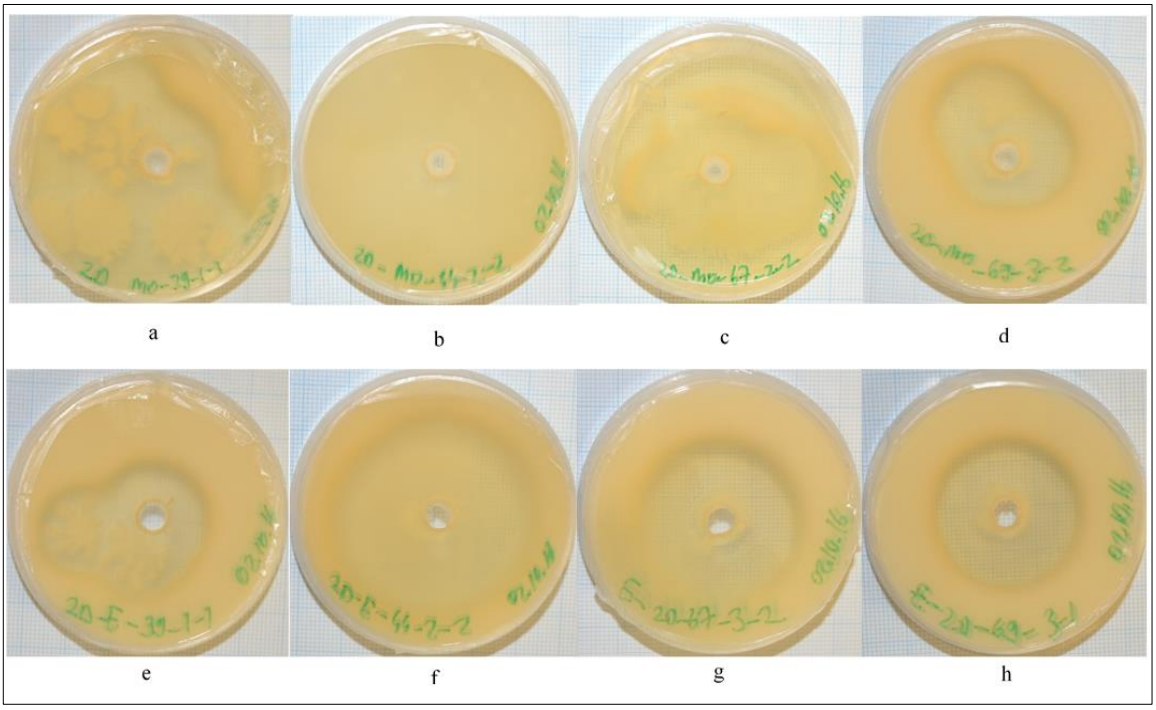

Figure 4.2. Skim milk agar clear zones of feather-induced isolated microorganisms a) 39 b) 44 c) 67 d) 69 and clear zones of this induced microorganisms supernatant e) 39 f) 44 g) 67 h) 69.

4.1.2. Identification Of Strain

To identify the microorganisms based on their FAME profile, Microbial Identification System (MIS) is used. Following FAME analysis of the isolate numbered 67, the strain was identified as *Bacillus sphaericus* and 69 numbered isolate was identified as *Bacillus cereus* by MIS. FAME profile of *Bacillus cereus* is given in **Figure 4.4**.

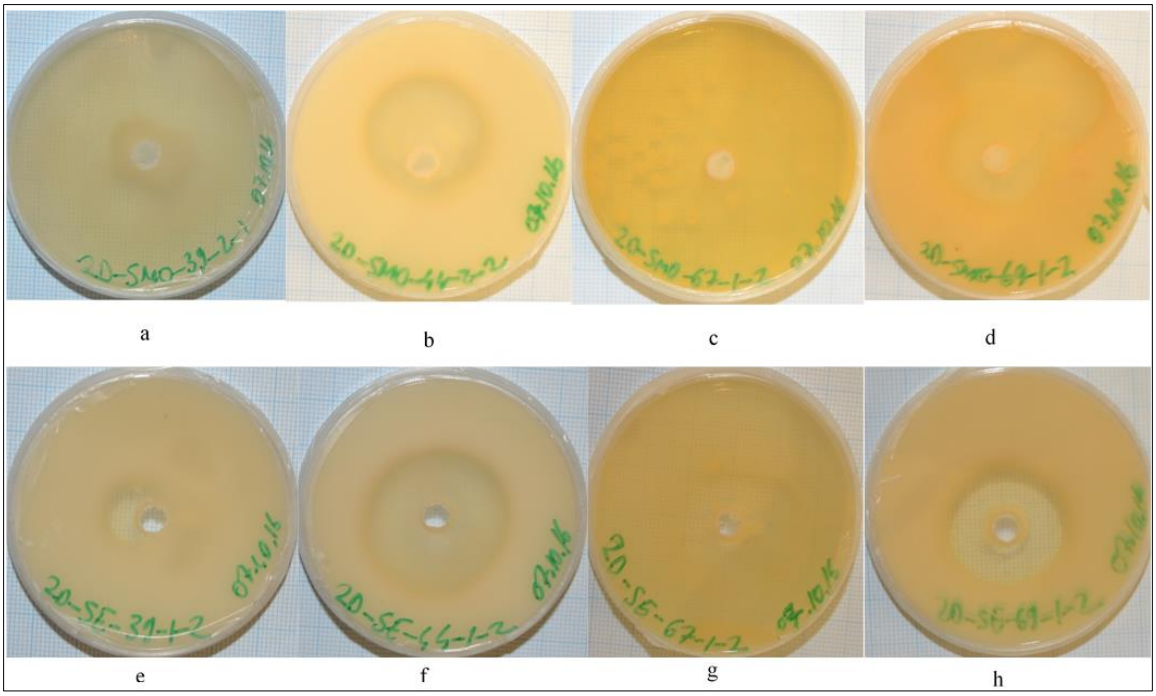

Figure 4.3. Skim milk agar clear zones of soy bean-induced isolated microorganisms a) 39 b) 44 c) 67 d) 69 and clear zones of this induced microorganisms supernatant e) 39 f) 44 g) 67 h) 69.

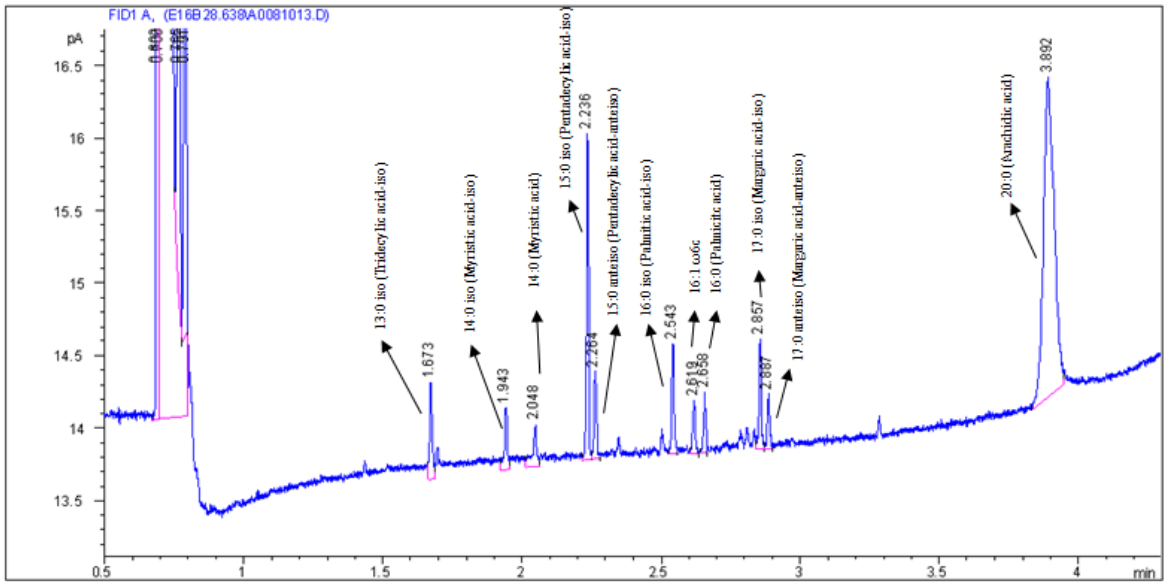

Figure 4.4. MIS analysis result as FAME profile for *Bacillus cereus* isolate number 69.

4.1.3. Growth Curve of *Bacillus cereus*

Bacillus cereus was grown in TSB (media containing 2.5 g/L glucose) with 10% inoculum at 150 rpm 37°C. Growth curve and glucose consumption is given in **Figure 4.5**. As seen in Figure **4.5**. glucose is finished around 6th hour. Organism is in stationary phase after 8th hour.

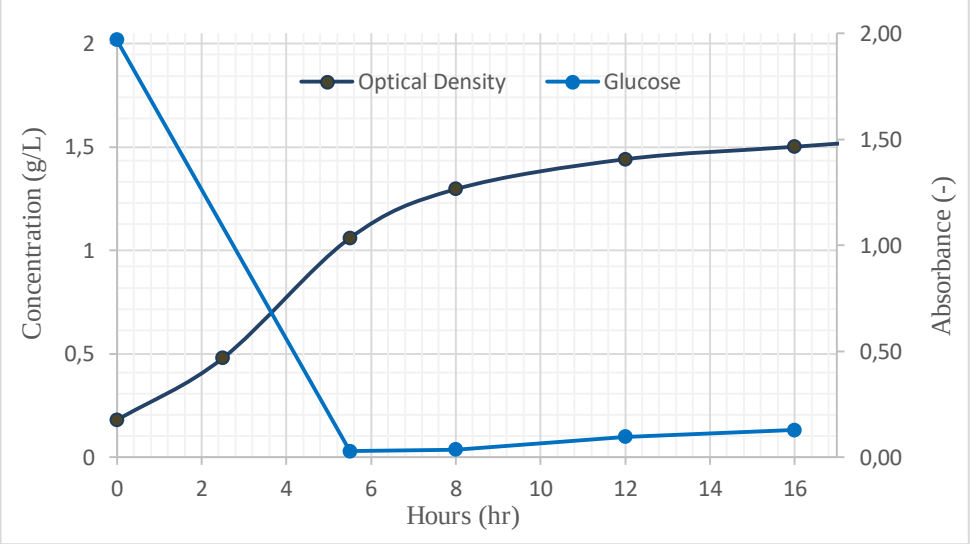

Figure 4.5. Growth curve of *Bacillus cereus*

4.2. KERATINOLYTIC ACTIVITY

4.2.1. Feather Degredation

To observe the degradation change of feather over days, *Bacillus cereus* was inoculated at 20% to feather containing medium and incubated at 37°C 150 rpm (Final working volume of 50 mL with 10g/L of feather concentration for each Erlenmeyer). As seen it **Figure 4.6** activity is increasing each day with a maximum increase in the second day. Negative blank which does not contain microorganism has 28.7% degradation (37°C, 150 rpm, 3 days). Visual change of before and after degradation (for 2 days of incubation) is given in **Figure 4.9**. Every day, total protein content of degraded feather was measured. The protein content is found to be (g/L): 0h, 0.3381; 1st day, 0.2230; 2nd day, 0.3243; 3rd day, 0.7363. Along with

degradation, soluble protein in solvent is rising (feather is not soluble in water due to its keratin content).

Using different inoculum size (between 2-20%) have indicated that doesn't have any significant effect on feather degradation in 2d as shown in **Figure 4.7**.

Activity saturated after 40 g/L when different concentration of feathers have been studied for degradation as shown in **Figure 4.8**.

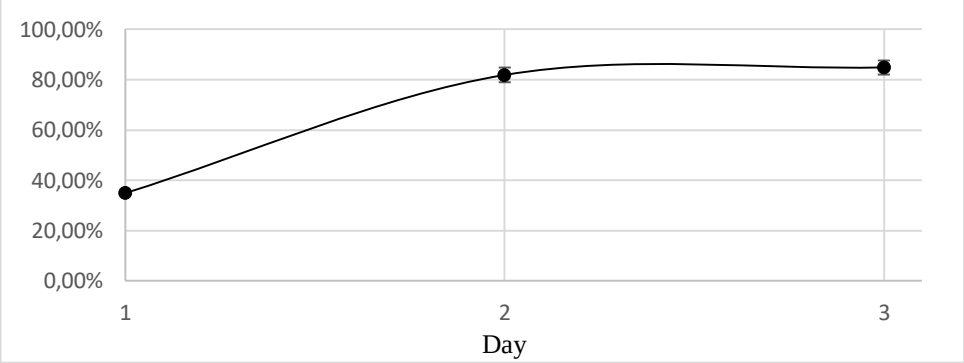

Figure 4.6. Percent degradation of *Bacillus cereus* over days (37°C, 150 rpm; 50 mL wvol, 10 g/L feather; Blank 28.71% degradation)

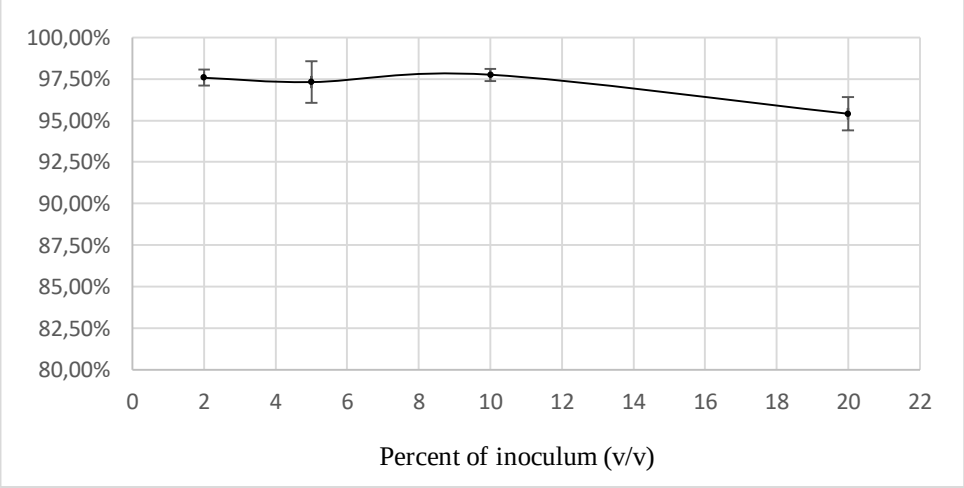

Figure 4.7. Percent degradation of *Bacillus cereus* with different inoculum size between 2-20% (37°C, 160 rpm, 2d, and 15 g/L feather)

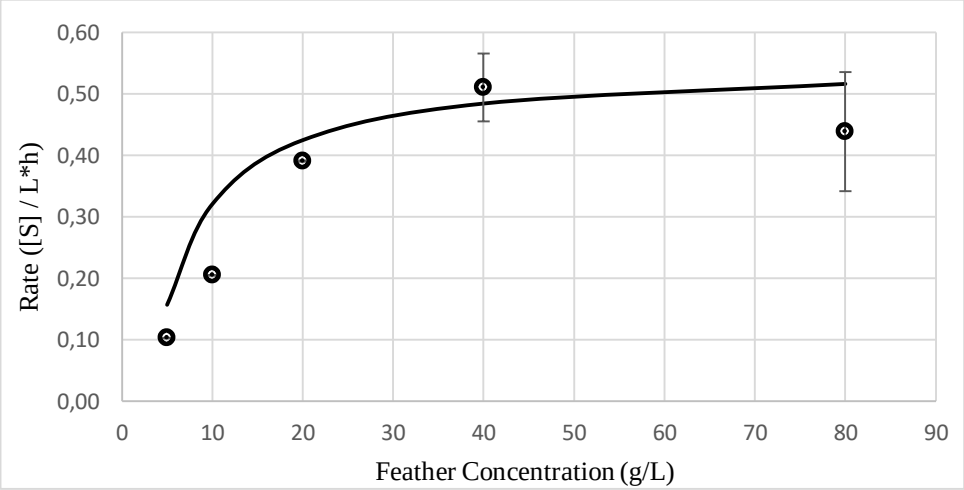

Figure 4.8. Overall Keratinolytic Activity of microorganism on degradation of feather with different concentrations (Inoculum size 10%, 37°C 160 rpm, 2d)

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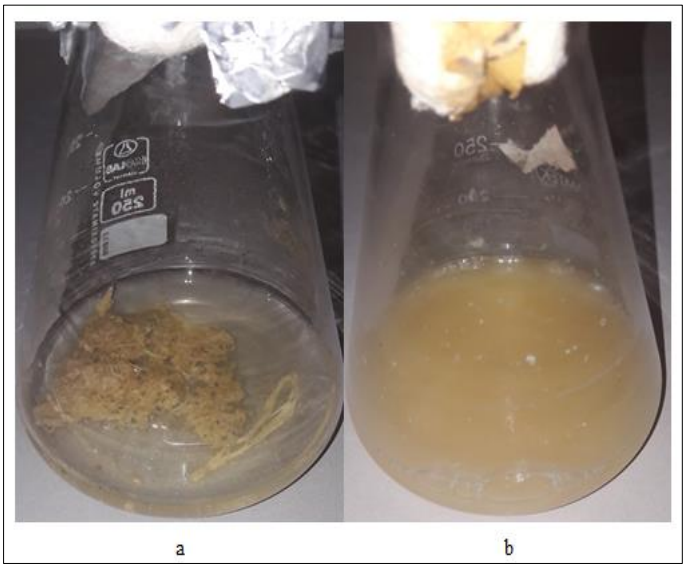

Figure 4.9. Feather, a) before degradation b) after degradation (Inoculum size 10%, 37°C 150 rpm, Incubation 2 days)

Degradation of whole feather is observed as shown in **Figure 4.10** and **Figure 4.11**. To evaluate mechanical effects, shaking conditions are 0 rpm (once-a-day swirled) and 100 rpm for each experiment. The experiments have whole feather in; water as a negative blank, commercial enzyme as a positive blank, non-induced microorganism, induced microorganism, and supernatant of induced-microorganism. Microorganisms were inoculated with the size of 20% to 50 mL wvol.

Negative blank is just distilled water. Positive blank is 50 μL commercial protease added to 50 mL wvol of medium. Non-induced microorganism just contains microorganism that is grown in TSB medium. Induced microorganism implies that microorganism is grown in TSB and then inoculated to feather containing medium for to be induced. Supernatant is collected from induced culture. This supernatant consists all wvol of whole feather degradation in its group. The whole feathers were not autoclaved.

Commercial enzyme, as expected, showed better activity than other groups for both shaken and non-shaken experiments. Negative blank didn't show any degradation. Non-induced microorganisms didn't show any degradation. Induced microorganisms have shown breakaways of barbs for shaken and non-shaken erlenmeyers. Supernatant have shown activity less than induced-microorganism in 2 days.

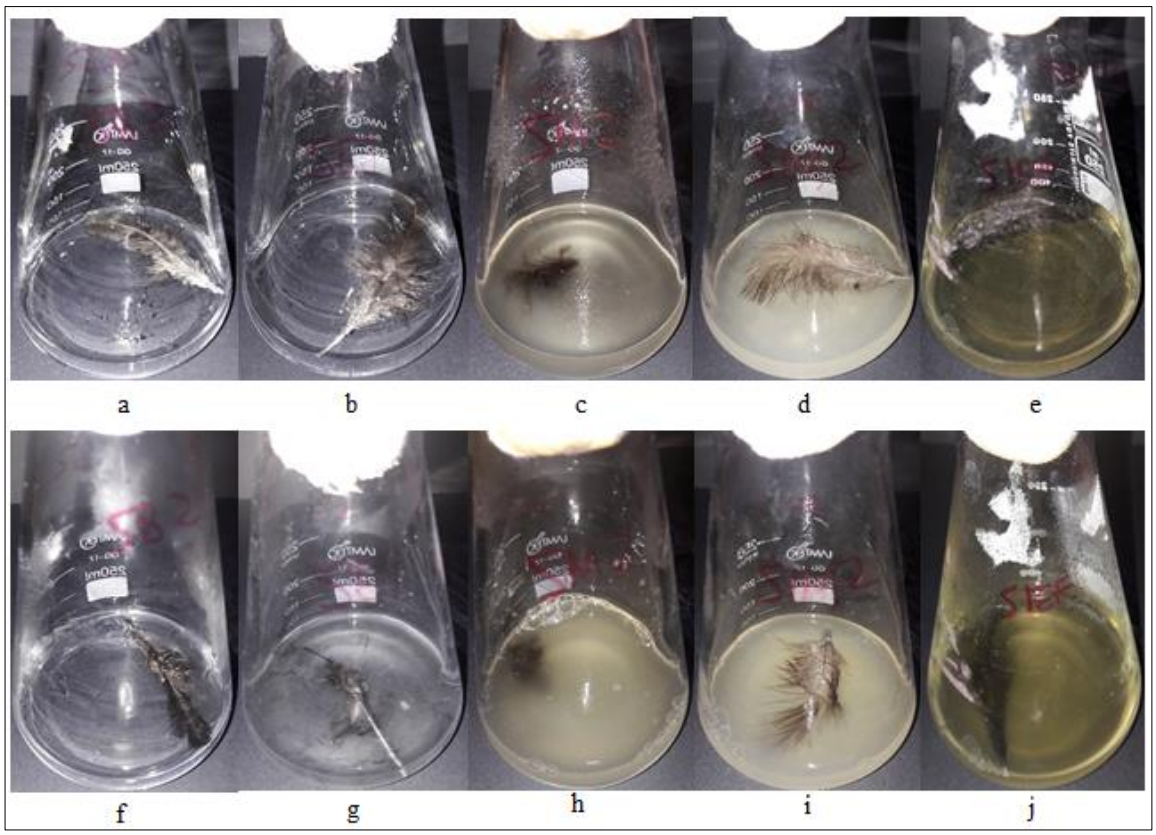

Figure 4.10. Whole feathers at 0th hour inside, a) just water as negative blank b) 50 µL commercial protease as positive blank, c) non-induced *Bacillus cereus* d) feather-induced *Bacillus cereus* and e) supernatant of induced broth. Results after two days inside, a) just water as negative blank b) 50 µL commercial protease as positive blank, c) non-induced *Bacillus cereus* d) feather-induced *Bacillus cereus* and e) supernatant of induced broth. Erlenmeyers are swirled just once-a-day manually. Incubation temperature is at 37°C

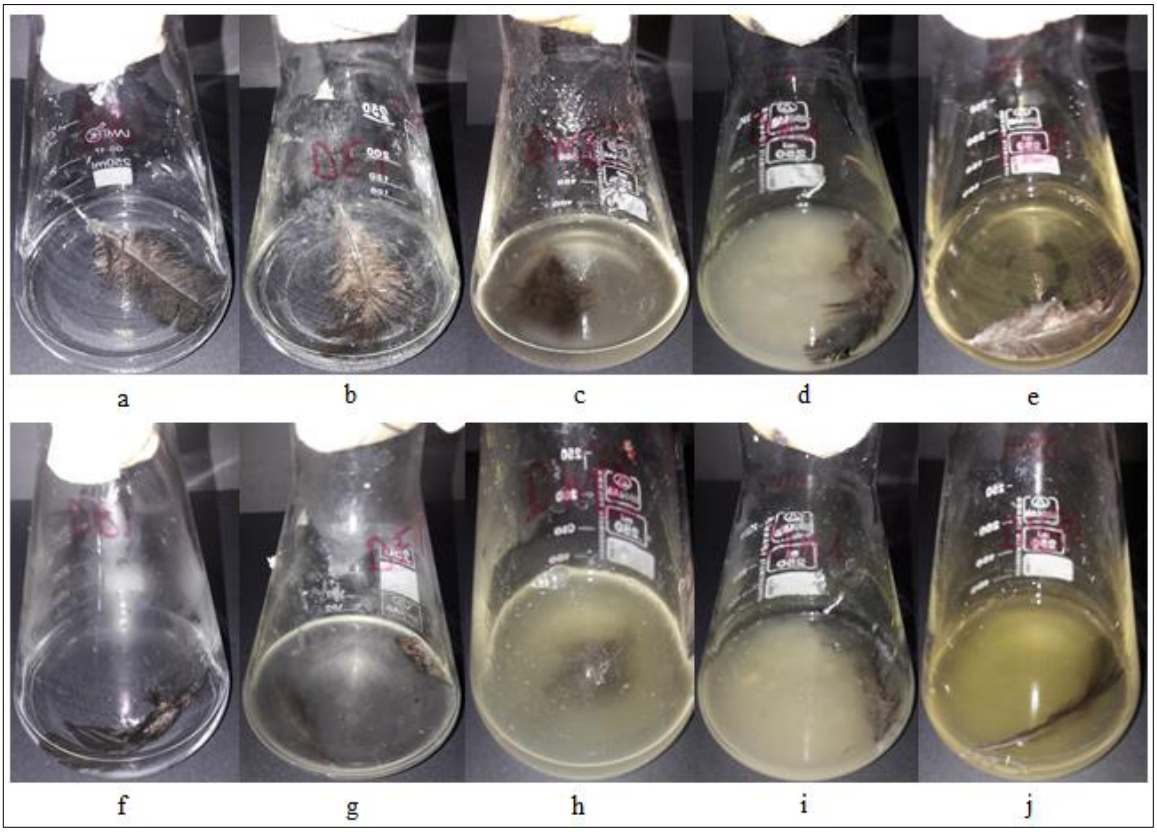

Figure 4.11. Whole feathers at 0th hour inside, a) just water as negative blank b) 50 µL commercial protease as positive blank c) non-induced *Bacillus cereus* d) feather-induced *Bacillus cereus* and e) supernatant of induced broth. Results after two days inside, a) just water as negative blank b) 50 µL commercial protease as positive blank c) non-induced *Bacillus cereus* d) feather-induced *Bacillus cereus* and e) supernatant of induced broth. Erlenmeyers are shaken just once a day manually. Erlenmeyers are shaken continously at 37°C 100 rpm for 2 days.

4.2.2. CHARACTERIZATION OF ENZYME

4.2.2.1. Enzyme Activity

Microorganisms are induced with feather and incubated for 3 days. Broth is then centrifuged and supernatant is collected for enzyme precipitation with ammonium sulfate. The precipitation was performed using 200 mL for 3 different concentration of ammonium sulfate: 60%, 70% and 80% (saturation). Ammonium sulfate is added at 4°C and precipitation was performed by stirring solution and later centrifuging it at 10000g for 10 min. After discarding the supernatant, the resulting pellets were dissolved in 40 mL Tris buffer. Then they were transferred into dialysis bags and left overnight with same Tris-HCl as surrounding buffer at 4°C. After dialysis, the final volume increased to 50 mL such that, considering the initial volume of 200 mL, the enzyme samples became theoretically 4 fold denser, and more active.

The precipitated samples were tested for their keratinolytic activity with commercial substrate named, keratin azure, releasing blue colour upon keratinolytic or proteolytic activity. Highest activity was observed with the sample treated with 80% ammonium sulfate (**Table 4.1**). Comparing with crude enzyme the activity is 2.5 times higher. The protein content was measured only for the samples treated with 80% ammonium salt. Protein content at 3rd day of this crude enzyme was determined and found 0.7363 g/L and 80% saturated was 2.0675 g/L.

Table 4.1 Enzyme activity of same samples with different conditions

	Enzyme activity (U/mL)	
Used blank	**10mM Tris-HCl as blank**	**Boiled samples as blank**
Crude enzyme	1.68	0.21
Precipitated using 60% $(NH_4)_2SO_4$	3.66	1.87
Precipitated using 70% $(NH_4)_2SO_4$	3.69	1.50
Precipitated using 80% $(NH_4)_2SO_4$	4.22	2.20

Lastly, the effect different substrate concentrations as well as different pH levels on the enzyme activity have been measured and presented in **Figure 4.12** and **Figure 4.13** respectively.

Using the kinetic data in **Figure 4.12**, the kinetic parameters of the enzyme, namely K_m and V^{max} values from Michaelis-Menten equation is also calculated using Lineweaver-Burk plot approach, followed by manual tuning of the parameters. The data in **Figure 4.12** yielded K_m= 28.0 g/L and V^{max}=35764.5 U/L.

Microorganism's degredation of feather substrate at different concentrations is shown in **Figure 4.8**. Activity has K_m of 5.2g/L and V^{max} of 0.55 gFeather/L/hr.

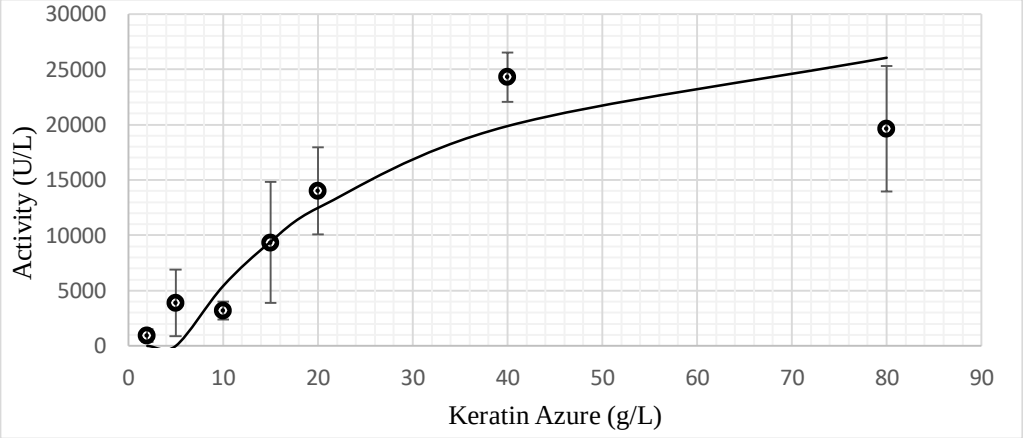

Figure 4.12 Keratinolytic Activity from Keratin Azure Concentrations.

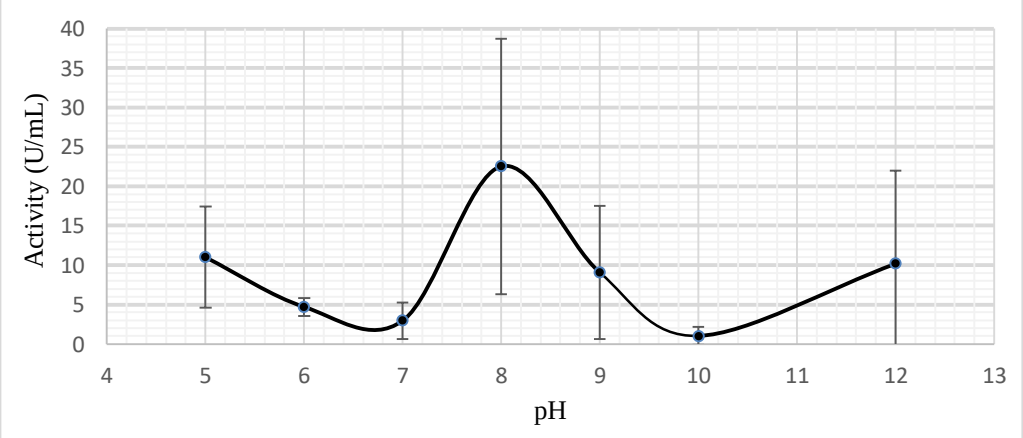

Figure 4.13 Keratinolytic Activity change over pH on Keratin Azure.

4.2.2.2. SDS-PAGE

To determine the molecular weight of the enzyme SDS-Page gel analysis was performed. There are seven significant bands, presented in **Figure 4.14**. Two bands between 15-25 kDa, two bands between 35-40 kDa, one band between 55-70 kDa, one band between 70-100 kDa, and one band between 100-130 kDa. Denser bands pointed that ammonium sulfate precipitation was successful

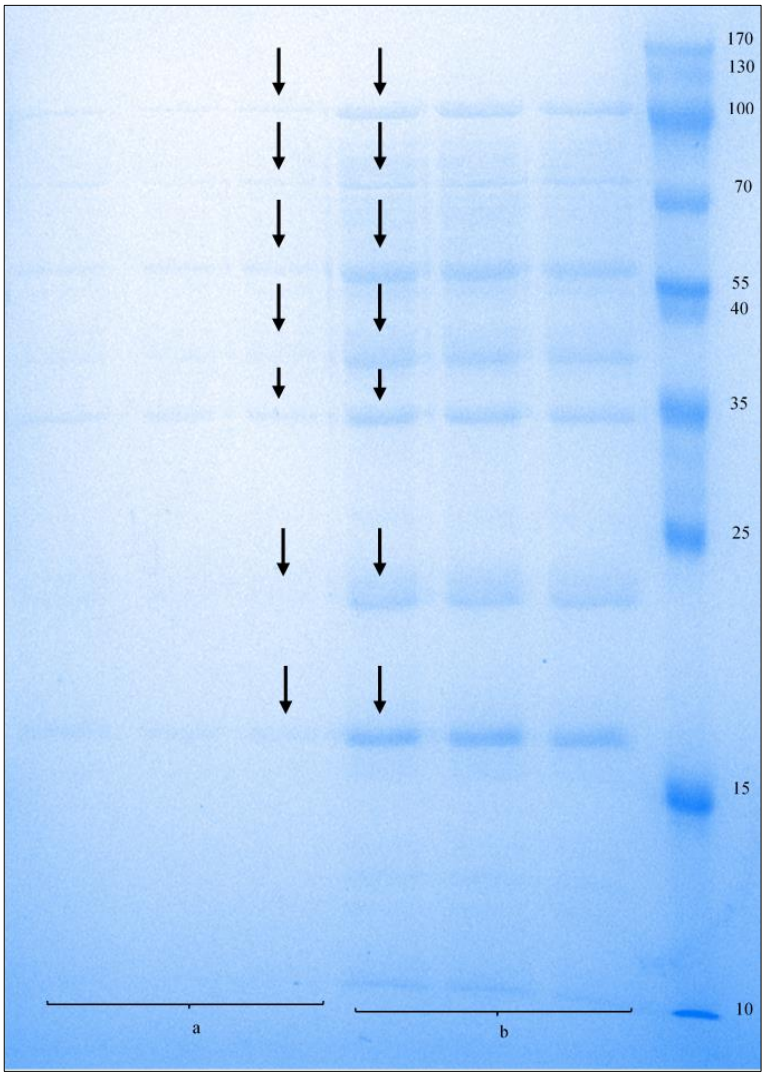

Figure 4.14. SDS-PAGE hace been made to a) crude enzyme b) 80% saturated with ammonium sulfate.

4.3. HPLC

To inspect of the nutritional value of the feather meal, the amino acid content of feathers have been quantified. For this study, the feathers are degraded with commercial protease enzyme and the hydrolysate was centrifuged, filtered and fed to the HPLC system. The results of this analysis as amino acid profile of the degraded feather is given as HPLC chromatogram are presented in **Figure 4.15** and the corresponding amino acid concentrations are given in **Table 4.2**.

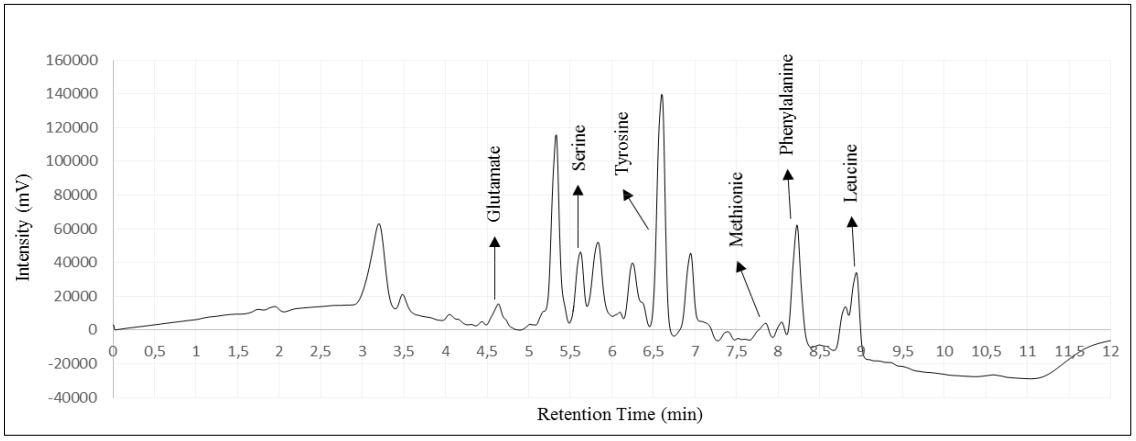

Figure 4.15. Amino Acid content chromatogram of feathers that are studied.

Table 4.2. The concentrations of selected amino acid from feather hydrolysate, analysed by HPLC

Amino Acid	Retention Time (m)	Concentration (g/L)
Glutamic acid	4.631	0.1377
Serine	5.619	0.0952
Tyrosine	6.598	0.1591
Methionine	8.038	0.0176
Phenylalanine	8.223	0.0862

5. DISCUSSION

Production of keratinase enzyme have some challenges. Chicken feather contains keratin, hydrophobic in nature, making the culture highly heterogeneous. Additionally, autoclaving the feather causes agglomeration of the feather in the medium, creating a "fur-ball" in the culture. These cause, in turn, large standard deviation among the replicates during feather degradation experiments.

The main challenge of getting the organism that has the highest activity is to narrow isolates. It have seen that isolates which are gathered from saline solutions on longer incubation times (isolates numbered between 61-70), has showed better proteolytic activity on skim milk than isolates between 29-44. Following this, isolates 39, 44, 67, 69 have been chosen for same (with some changes on experiment which is explained below) skim milk clear zone activity experiments to observe proteolytic activities. They were enriched/induced in feather and soy bean containing media, separately. Broth itself containing microorganisms and their supernatant is used for clear zone experiments.. 67 and 69 showed better activity again as stated above. In MIS analysis 67 and 69 were identified and named as *Bacillus sphaericus* ENKK-67 and *Bacillus cereus* ENKK-69. *Bacillus cereus* was chosen for slightly better skim milk clear zone activity.

Bacillus cereus is gram positive, rod shaped, facultative aerobic, mesophilic, endospore forming bacteria. Usually it is found in soil. Being gram positive and mesophilic has advantages for enzyme production. As keratin is a component that can't be digested inside cell easily, enzymes should have been sent outside of cell. In purification and separation operations extracellular enzymes decreases the downstream operation cost and the enzyme doesn't get damaged with extra treatments. Also this bacteria grows in temperatures between 20-40°C. Lower enzyme production temperatures are important for energy costs in large-scale productions.

Bacillus cereus enters to stationary phase at 8^{th} hour and glucose depletes at 6^{th} hours in TSB. In same conditions this microorganism is inoculated on feather containing medium at 16^{th}-18^{th} hours. So organism is around 10 hours without any glucose. In further studies inoculum hour should be studied for optimization of shortening total process hours and gain maximum activity.

Degradation of feather is not high in first day. Highest degradation rate occurs between first and second day. Because of this all keratinolytic activity experiments were performed with enzymes (supernatant) that are collected on 2nd day. Inoculum size didn't show any significant effect on degradation. If results would have been gathered on first day (experiment results were gathered on second day), degradation percentage may significantly vary. After full medium optimization, first day's activity could be considered for comparison. Feather concentrations, effects the degradation percentage, significantly, from 40 g/L and higher concentrations comparing below 40 g/L. It may be reasoned as the enzyme activity saturates after 40 g/L. On the other hand the reason could be that microorganisms work better on suspension which allows to enzymes to distribute homogenically. After 40 g/L, feather does not allow proper shaking in Erlenmeyers. Problem could be solved with increasing the inoculum size.

As expected, commercial protease showed better activity than others on whole feather degradation. Non-induced microorganism, didn't show any activity on whole feather degradation. Induced microorganism showed expected activity with breaking away the barbs in this experiment but their supernatant, which contains the enzyme that is expected to be extracellular, didn't showed good degradation as induced-microorganisms itself. It is thought that it may need more time. Also continuously shaken Erlenmeyers showed better degradation than once-a-day swirled Erlenmeyers (only for commercial protease, induced-microorganism, and supernatant) which was expected for providing homogeneity for microorganisms and enzymes in the culture.

Ammonium sulfate precipitations were performed in different saturations. 80% saturated supernatant showed higher enzymatic activity than others. 80% saturation was used for precipitation for supernatants on other activity experiments.

Keratinolytic activity of crude enzyme was increased with ammonium sulfate precipitation, dissolving pellet in lower volume from beginning, and with following dialysis. But the increase activity have been expected at least 4 folds. But maximum increase was 2.5 fold. So this yield loss should have been investigated with further studies. Loss was not just on activity but also with total protein content which in large scale would be important. This could be improved with making membrane dialysis two days beside one with changing the surrounding buffer each day. Also changing molarity and pH of the buffer may affect the activity. Even though the denser protein content and higher enzyme activity is not as

expected, the separation results are successful as it can be seen in keratinase activity, total protein content, and SDS-PAGE experiments.

The enzyme, showed highest activity on pH 8 which shows that it is a alkaline protease. Second high activity was at pH 5. It may be reasoned as, acidic environment itself (not enzyme) degrades keratin azure and releases blue dye. This situation could affected the activity measurement results. In contrast there may be several proteases which one of them works in acidic environment.

Effect of the substrate concentration experiment, after 40g/L it shows slight decrease which means activity is saturated. Beside saturation of the activity, after 40 g/L substrate could not be allowing proper mixing of enzyme (activity experiment is in shaken water bath). Same problem occurred on feather degradation with microorganism. Cutting keratin azure in small pieces could, at least, help for lower standard deviations.

Keratinolytic activity measurements with keratin azure is important for comparison with other studies. Activity measurements are performed also with chicken feather. But chicken feather's structure can differ slightly from chicken species to others and from it parts such as barbs, rach, calamus. So comparison with other studies is not easy to be standardized. Keratin azure is right choice for comparasion. It has simple colorimetric way for activity measurement because it releases blue dye to solvent.

SDS-PAGE bands are in expected range which is mentioned in literature. Changes in bands density shows that ammonium sulfate precipitation was successful. Bands density and clarity could be improved with different staining techniques such as silver staining. Reasons for many bands could be explained as they are subunits of one enzymes or subunits of more enzymes which can be isozymes. Apart from these it could be just one enzyme and polypeptide chains from keratin which has more possibility than others fore mentioned. These possibilities could be analysed with further studies such as separating them with size exclusion chromatography and measure the activity of each band.

Standards, in chromatogram, were showing good results separately. Beside the amino acids that are given in the results, there were three more amino acids (tryptophan, leucine, glycine) that gave good peaks (totally 8 amino acid were studied) while making standard curves of areas. In our runned sample they couldn't be read possibly because of some interactions between them. Some modifications were done in gradient, flow rate but they didn't show

better peaks. Also chromatogram line is going below 0 mV after 7th minute. Harsh shifting between eluents (gradients) or using less polar eluent than methanol could solve problem. In further studies, other pre-column derivatization agents will be tested and some procedures that doesn't contain derivatization will be performed to observe peaks with better quality.

Summing up, with determination of better optimum production conditions, and with better separation and purification techniques and conditions higher activity will be observed in future studies.

6. CONCLUSION

This aim of this study is to isolate and characterize a microorganism with keratinolytic activity. The isolated microorganism, identified as *Bacillus cereus* species based on fatty acid profile of the crude extracts. The strain is tentatively named as *Bacillus cereus* ENKK-69, and the extracellularly produced keratinase enzyme have been characterized for its kinetic parameters and molecular weight. Operating conditions for production, pH, inducer concentration, growth rate is also determined.

The keratinase enzyme that is produced from our isolate has shown to be industrially useful. Additionally, downstream methods have been successfully applied to purify the enzyme, thereby increasing its activity.

Further studies should focus on medium optimization for improved production, scale-up strategies, identification of the strain using molecular methods (e.g. 16S sequencing) and determination of co-factors/inhibitors of the enzyme as well as substrate specificity to further characterize the enzyme.

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