UNIVERSITI PUTRA MALAYSIA

ISOLATION, PRODUCTION AND CHARACTERIZATION OF KERATINASE FROM *Bacillus* Sp. Khayat

MOHD EZUAN BIN KHAYAT

FBSB 2011 37
ISOLATION, PRODUCTION AND CHARACTERIZATION OF KERATINASE FROM Bacillus Sp. Khayat

By

MOHD EZUAN BIN KHAYAT

Thesis is Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Sciences

February 2011
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of
the requirement for the degree of Master of Science

ISOLATION, PRODUCTION AND CHARACTERIZATION OF
KERATINASE FROM *Bacillus* Sp. Khayat

By

MOHD EZUAN BIN KHAYAT

February 2011

Chairman : Associate Professor Mohd. Yunus Abdul Shukor, PhD
Faculty : Biotechnology and Biomolecular Science

The increase in demand of chicken meat products for human consumption has caused
the accumulation of feather waste. In this research, seven local feather degrading
bacteria have been isolated from soil and feather waste samples around Selangor and
Johor, Malaysia. All the bacteria that obtained from the sampling procedure were then
screened by incubating them in basal media that contained feathers as a carbon and
nitrogen sources. Among the isolates, isolate E3 has shown the highest keratinolytic
activity and feather degradation percentage compared to the others. Isolate E3 was
then identified as *Bacillus* sp. khayat based on its 16s rRNA sequences. This strain
produced keratinase optimally at a temperature of between 30 to 37°C and at pH 8.
The optimal temperature and pH for the bacterial growth were also found at 30 to 37°C
and at pH 7.5 to 9 respectively. Studies using different carbon sources on keratinase
production also showed that the addition of skim milk has enhanced enzyme
production. The optimum concentration of skim milk for keratinase production was
found at 0.2 gL⁻¹. The concentration of feather for optimum keratinase production was
determined at 1% (w/v) while for optimum growth at 0.5 to 1.5% (w/v). The bacterium was able to degrade up to 82.43% of feather in seven days with the highest keratinase production observed at the third day of incubation period. The keratinase enzyme from the bacterium was then purified through anion exchange chromatography, using DEAE cellulose as a matrix, and gel filtration chromatography, using Zorbax® column. The molecular weight analysis using SDS-PAGE gel revealed that the enzyme has a molecular weight of approximately 31.62 kDa. The optimum temperature and pH of the enzyme activity were 40°C and pH 8 respectively. This enzyme can also retain over 80% of its original activity for one hour when preincubated at temperatures of between 20 to 45°C and at pH 6.5 to 10.

In the protease inhibitor study, the enzyme was greatly inhibited by the addition of PMSF compared to other inhibitors indicating that the enzyme is a serine type protease. The enzyme was also observed to be inhibited by the presence of all tested reducing agents such as DTNB, DTT, and 2-marcaptoethanol. All of tested metal ions such as Zn²⁺, Hg⁺, Ag⁺, Pb²⁺, Mg²⁺, Cu⁺, K⁺, Co²⁺, and Ca²⁺ were found to give negative effect on keratinase activity. This keratinase was active against various types of proteinous substrates either keratonious or unkeratinous proteins. However, the highest activity was observed when casein was used as the substrates, followed by soluble keratin, BSA, egg albumin, feather, wool, and human hair. The results of this study are very importance since they can be used to raise the potential of keratinase from Bacillus sp khayat in industrial applications.
Abstrak tesis yang dikemukan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENCILAN, PENGHASILAN DAN PENCIRIAN KERATINAS DARIPADA Bacillus Sp. Khayat

Oleh

MOHD EZUAN BIN KHAYAT

Februari 2011

Pengerusi : Profesor Madya Mohd. Yunus Abdul Shukor, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Peningkatan permintaan orang ramai terhadap produk-produk berdasarkan daging ayam telah menyebabkan akumulasi sisa bulu ayam. Dalam kajian ini, tujuh bakteria pengurai bulu ayam telah dipencilkan daripada sampel tanah dan sisa bulu ayam di sekitar Selangor dan Johor, Malaysia. Kesemua bekerja yang diperolehi dari prosedur penyampelan kemudiannya disaring dengan dieramkan di dalam media asas yang mengandungi bulu ayam sebagai sumber karbon dan nitrogen. Dikalangan isolat tersebut, isolat E3 telah didapati mempunyai aktiviti keratinas dan peratusan penguraian bulu ayam tertinggi berbanding yang lain. Isolat E3 kemudiannya telah dikenalpasti sebagai Bacillus sp. khayat berdasarkan jujukan 16s rRNanya. Strain ini menghasilkan keratinase secara optima pada suhu 30 hingga 37°C dan pada pH 8. Pertumbuhan optima bakteria ini juga ditemui pada suhu diantara 30 hingga 37°C dan pada pH 7.5 hingga 9.0. Kajian menggunakan pelbagai sumber karbon juga menunjukkan penambahan susu skim ke dalam media meningkatkan penghasilan keratinase. Kepekatan optima susu skim untuk penghasilan keratinase tersebut ialah
0.2 gL⁻¹. Kepekatan bulu ayam untuk penghasilan optima keratinas juga telah ditemui pada 1% (w/v) manakala untuk pertumbuhan bakteria adalah pada kepekatan 0.5 hingga 1.5% (w/v). Bakteria tersebut mampu meghuraikan bulu ayam sehingga 82.43% dalam 7 hari. Penghasilan keratinas tertinggi pula ialah pada hari ketiga pengeraman. Keratinase daripada bakteria tersebut kemudian telah ditulenkan menggunakan kromatografi anion, dengan DEAE sellulosa sebagai matrik, dan kromatografi penurasan gel, menggunakan kolum Zorbax®. Analisis berat molekul menggunakan gel SDS-PAGE menunjukkan enzim tersebut mempunyai berat molekul lebih kurang 31.62 kDa. Suhu dan pH optimum untuk aktiviti enzim tersebut adalah masing-masing pada 40°C dan pH 8. Enzim ini dapat megekalkan aktiviti asalnya lebih daripada 80% apabila dieram dalam suhu 20 hingga 45°C dan pH 6.5 hingga 10. Dalam kajian terhadap kesan perencat protease, aktiviti enzim ini telah direncat dengan banyaknya oleh PMSF dan ini menunjukkan ia tergolong dalam serine proteas. Aktiviti enzim ini juga didapati terencat dengan kehadiran kesemua agen-agen penurunan yang dikaji seperti DTNB, DTT, dan 2-marcaptoethanol. Kesemua ion logam yang dikaji seperti Zn²⁺, Hg⁺, Ag⁺, Pb²⁺, Mg²⁺, Cu⁺, K⁺, Co²⁺, dan Ca²⁺ telah menunjukan kesan negatif terhadap aktiviti keratinas. Keratinas ini aktif terhadap berbagai-jenis substrat protein sama ada protein keratin atau bukan keratin. Akan tetapi, aktiviti tertinggi telah diperhatikan apabila kasein digunakan sebagai substrat, diikuti oleh larutan keratin, albumin telur, bulu ayam, bulu kambing, dan rambut manusia. Hasil kajian ini adalah amat penting kerana dapat meningkatkan lagi potensi keratinase dari Bacillus sp khayat dalam aplikasi industri.
ACKNOLEDGEMENT

My sincere appreciation goes to my supervisor, Assoc. Prof. Dr. Mohd Yunus Abd. Shukor for his endless advice, patience, and encouragement that has led to the completion of this research.

I also would like to convey my deepest appreciation to my committee member, Prof. Dr. Mohd Arif Syed for his supervision, advice, constructive suggestion, and for reviewing my works during the period of this study.

My warmest gratitude also goes to all my lab mates, Mr. Badrin, Mr. Haris, Mr. Afif, Mr. Zaquan, Mr. Baskaran, Mr. Zaki, Ms. Norliza, Ms. Inaz, Ms. Ku Nurul, Ms. Huda, and all undergraduate and postgraduate students at department of Biochemistry for their assistances and supports.

Last but not least, I would like to take this opportunity to thank all my family, my mother, late father, and sisters for being there when I need them most. I would also like to extend my deepest gratitude to Ms. Norain Mohd Tamsir for giving me inspiration and support to archive my dreams.
I certify that an Examination Committee has met on date of viva voce to conduct the final examination of Mohd Ezuan Khayat on his Master of Science thesis entitled “Isolation, Production, and Characterization of Keratinase from Bacillus sp. khayat” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Putra Malaysia (Higher Degree) Regulation 1981. The committee recommends that the student be awarded the degree of Master of Science.

Members of Examination Committee were as follows:

Name of Chairperson, PhD  
(Professor/ Associate Professor/ Ir)  
Name of Faculty  
Universiti Putra Malaysia  
(Chairman)

Name of Examiner 1, PhD  
(Professor/ Associate Professor/ Ir)  
Name of Faculty  
Universiti Putra Malaysia  
(Internal Examiner)

Name of Examiner 2, PhD  
(Professor/ Associate Professor/ Ir)  
Name of Faculty  
Universiti Putra Malaysia  
(Internal Examiner)

Name of External Examiner, PhD  
(Professor/ Associate Professor/ Ir)  
Name of Department and/or Faculty  
Name of Organisation (University/ Institute)  
(External Examiner)

________________________________________

BUJANG KIM HUAT, PhD

Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
This thesis was submitted to the senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Mohd. Yunus Abd. Shukor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd. Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

______________________________

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

MOHD EZUAN KHAYAT
Date: 21 February 2011
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>ABSTRAK</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>AKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>APPROVAL</td>
<td>vii</td>
</tr>
<tr>
<td></td>
<td>DECLARATION</td>
<td>ix</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td></td>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

### CHAPTER

1. **INTRODUCTION**

2. **LITERATURE REVIEW**
   
   2.1 Feather
   2.2 Keratin
      
      2.2.1 Structure of keratin
      2.2.2 Stability of keratin
   2.3 Potential of recycling keratinous waste
      
      2.3.1 Animal feed
      2.3.2 Fertilizer
      2.3.3 Film and coating
   2.3 Keratin degradation in nature
      
      2.3.1 Keratinolytic bacteria
      2.3.2 Keratinolytic actinomyces
      2.3.3 Keratinolytic Fungi
   2.4 Mechanism of microbial keratinolysis
      
      2.4.1 Mechanical keratinolysis
      2.4.2 Sulphitolysis
      2.4.3 Proteolysis
   2.5 Cultural condition of microbial keratinase production
      
      2.5.1 Effect of pH
      2.5.2 Effect of temperature
      2.5.3 Effect of addition of carbon source
      2.5.4 Effect of addition of nitrogen source
      2.5.5 Substrate
   2.6 Characteristics of keratinase
      
      2.6.1 Optimum temperature
      2.6.2 Optimum pH
      2.6.3 Molecular weight
      2.6.4 Substrate specificity
      2.6.5 Effect of protease inhibitor
      2.6.6 Effect of metal ion
2.7 The application of keratinase
  2.7.1 Improvement of animal feed value
  2.7.2 Use in leather industry
  2.7.3 Degradation of prion protein
  2.7.4 Detergent application
  2.7.5 The other application of keratinase

3 MATERIALS AND METHODS
  3.1 Equipments and chemicals
  3.2 General methods
    3.2.1 Feather basal salt media
    3.2.2 Skim milk agar
    3.2.3 Saline phosphate buffer
    3.2.4 Preparation of chicken feather
    3.2.5 Synthesis of azokeratin
    3.2.6 Keratinase assay
    3.2.7 Determination of feather degradation
  3.3 Sampling and Screening of feather degrading bacteria
    3.3.1 Soil sampling
    3.3.2 Isolation of keratinolytic microorganism
    3.3.3 Screening of keratinolytic bacteria
  3.4 Identification of keratinolytic bacterium
    3.4.1 Gram staining test
    3.4.2 Molecular characterization of keratinolytic bacterium
      3.4.2.1 Genomic DNA extraction
      3.4.2.2 Amplification of genomic DNA by using Polymerase Chain Reaction (PCR)
      3.4.2.3 Purification of Amplified PCR products
      3.4.2.4 Detection of PCR products
      3.4.2.5 Phylogenetic analysis
  3.5 Optimization of keratinase production by Bacillus sp. Khayat
    3.5.1 Optimization of initial pH
    3.5.2 Optimization of temperature
    3.5.3 Optimization of carbon sources
    3.5.4 Optimization of co-nitrogen sources
    3.5.5 Optimization of skim milk concentration
    3.5.6 Optimization of feather concentration
    3.5.7 Time course study for growth and keratinase production by Bacillus sp. Khayat
  3.6 Purification of keratinase
    3.6.1 Preparation of crude keratinase
    3.6.2 Concentration of crude keratinase and buffer exchange
    3.6.3 Ion exchange chromatography using DEAE cellulose
    3.6.4 Gel filtration chromatography using Zorbax® GF 250
    3.6.5 Protein determination
  3.7 Characterization of keratinase
    3.7.1 Determination of molecular mass
    3.7.2 Staining and destaining
    3.7.3 Effect of pH on keratinase activity and stability
    3.7.4 Effect of temperature on keratinase activity and stability
3.7.5 Effect of protease inhibitors and reducing agents on keratinase activity  
3.7.6 Effect of metal ions on keratinase activity  
3.7.7 Substrate specificity of keratinase

4 RESULTS AND DISCUSSIONS

4.1 Isolation of feather degrading bacteria  
4.2 Screening of feather degrading bacteria  
4.3 Identification of isolate E3  
4.3.1 Gram staining  
4.3.2 16S rRNA analysis  
4.4 Optimization of keratinase production by Bacillus sp. khayat  
4.4.1 Optimization of initial pH  
4.4.2 Optimization of temperature  
4.4.3 Effect of carbon source on keratinase production by Bacillus sp. khayat  
4.4.4 Effect of nitrogen source on keratinase production by Bacillus sp. khayat  
4.4.5 Effect of skim milk concentration on keratinase production of Bacillus sp. khayat  
4.4.6 Effect of feather concentration on keratinase production of Bacillus sp. khayat  
4.4.7 Time course for growth, feather degradation and keratinase production of Bacillus sp. khayat

4.5 Purification of keratinase from Bacillus sp. khayat  
4.5.1 Concentration of crude enzyme  
4.5.2 Ion exchange chromatography  
4.5.3 Size exclusion chromatography

4.6 Properties of keratinase  
4.6.1 Molecular weight of keratinase  
4.6.2 Optimum pH for keratinase activity  
4.6.3 pH stability of keratinase  
4.6.4 Optimum temperature for keratinase activity  
4.6.5 Temperature stability of keratinase  
4.6.6 Effect of protease inhibitor and reducing agents on keratinase activity  
4.6.7 Effect of metal ions on keratinase activity  
4.6.8 Substrate specificity of keratinase

5 CONCLUSIONS

REFERENCES

APPENDIC A
APPENDIC B
BIODATA OF STUDENT

xii