UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF RECALCITRANT CHICKEN FEATHER WASTES BY LOCALLY ISOLATED HEAVY METAL TOLERANT Alcaligenes sp. STRAIN AQ05-001

IBRAHIM YUSUF

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By

IBRAHIM YUSUF

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my parents, family and friends for their never-ending prayers and blessings throughout my studies.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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Chairman : Siti Aqlima Binti Ahmad, PhD
Faculty : Biotechnology and Biomolecular Sciences

The global increase in consumption of chicken meats as source of protein and the current use of chicken feathers as source of cheaply available biosorbent materials has led to the generation of large volume of recalcitrant melanised and non-melanised keratin containing primary and secondary feathers wastes in our environments. A need for eco-friendly method of managing these wastes became highly necessary.

A novel bacterium isolated from feather dump site located in Johor, Malaysia identified by physical, biochemical and 16S RNA sequencing techniques as *Alcaligenes* sp. AQ05-001 was successfully used to degrade such feather wastes to produce keratinase enzyme and protein rich hydrolysates. Initially it only degraded 1 g/L of heavy metal polluted and un polluted feather wastes, produced 8.84 U/mL keratinase in 3 days. But upon optimisation of physical and nutritional factors using one-factor-at-a-time and response surface methodology approaches, complete degradation of almost 5 g/L feather and a 10-fold increase in keratinase production (88.0 ±1 U/mL) was recorded. The bacterium was immobilised in gellan gum gelling agent with its conditions optimised. With 0.8% gellan gum concentration, 3 mm size beads and 250 initial cell loads, immobilised cells degraded feathers at a faster rate and produced higher keratinase activities in optimised media. Both free and immobilised cells efficiently degraded recalcitrant melanised feathers faster than non-melanised feathers. They both showed a high preference for black feathers and complete degradation of 5 g/L of black feathers to dust and fibres was achieved in 18 and 24 h with immobilised and free cells, respectively. Interestingly, the strain further selectively degraded black feather completely in a flask containing 5 g/L mixture of equal amount of black, brown and white feathers, with leftover of brown feathers rachises at the end of 36 h incubation. Further, free and immobilised bacteria degraded large amount of feathers in optimised media. About 50 g/L feathers were degraded by immobilised cells while free cells degraded up to 40 g/L in 60 h with production of high amount of soluble protein hydrolysates that are rich in both essential and non-essential amino acids such as leucine, isoleucine, cysteine, aspartic acid and proline. Optimal adsorption of heavy metals by chicken feathers was carried out in broths within the pH range of 7-8.5 and

temperature between 25-35 ℃. Both free and immobilised cells degraded feather-laden with single or combination of heavy metals with concurrent production of higher keratinase enzyme at varying rates. Beads were reused in biodegradation of feathers polluted with different heavy metals in semi-continuous fermentation for a number of cycles ranging from 4-10. Encapsulated cells tolerated up to 30 ppm of Ag, 20 ppm of Co, 15 ppm of Cu, 10 ppm of As, Ni, Cd and 5 ppm of Hg, Pb while free-living cells tolerated only 1 ppm of Hg and Pb. Moreover, cells in gellan gum degraded about 95% and above of feathers polluted with a mixture of 5 ppm each of Ag, As, Cd, Co, Cu, 10 and 20 ppm each of Ag, Co and Cu at 18 h. Beads that failed to degrade feathers laden with higher concentration of heavy metals like mercury, were used to degrade feathers laden with other heavy metals for many cycles without need for mineral acid desorption. Crude keratinase produced by strain AQ05-001 displayed relative activity over a temperature ranging from 30-60 ℃, pH 7-10 and was highly inhibited by serine inhibitor, Tween20, Trixton-100 and sodium dodecyl sulphate. However, cell-free crude keratinase was not able to degrade feathers efficiently, but improved degradation was observed in the presence of reducing agent (2 mercaptoethanol).

The strain possesses the potential of being used in the management of melanised, non-melanised, heavy metal free/polluted feather as well as in the production of protein-rich hydrolysates for animal feeds and also in the industrial production of valuable keratinase enzyme. Furthermore, not only its heavy metals tolerability can be harnessed in the bioremediation of heavy metal-contaminated feather wastes, but it could also use the contaminated wastes as a substrate for keratinase production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doctor Falsafah

PENURUNAN SISA BUANGAN BULU AYAM YANG SANGAT DEGIL OLEH PEMENCILAN BAKTERIA TEMPATAN PELBAGAI KETAHANAN LOGAM BERAT - *Alcaligenes* sp. STRAIN AQ05-001

Oleh

IBRAHIM YUSUF

Mei 2016

Pengerusi : Siti Aqlima Binti Ahmad, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Peningkatan dalam pengambilan harian daging ayam dalam hidangan kita yang telah membawa kepada jumlah besar generasi multi buangan bulu ayam berwarna yang tahan protease konvensional atau serangan mikrob, dan dengan itu membentuk gangguan dalam alam sekitar. Di samping itu, penggunaan berterusan sisa bulu ini adalah semurah bahan-bahan bio penyerap dalam penyingkiran logam berat dari buangan industri dan air sisa serta pelupusan haram massa besar bulu di tempat-tempat di mana ia boleh menyerap logam, telah membawa kepada pengumpulan sisa bulu yang sangat sukar dan toksik yang menimbulkan isu biodegradasi serius. Sejak cara konvensional melupuskan sisa ini tidak mesra alam, keperluan untuk mengasingkan strain logam berat toleran yang berkesan boleh merendahkan bulu adalah sangat perlu. Pengasingan, pencirian dan pengoptimaan keadaan fizikal dan kultur strain baru bakteria yang mampu menggunakan kedua-dua bahan buangan pertama dan kedua sebagai substart untuk menghasilkan hidrolisat yang kaya keratinase and protein dilaporkan. Bakteria telah dikenal pasti secara fizikal, bikimia dan jujukan 16S RNA sebagai *Alcaligenes* sp. AQ05-001. Ini kali pertama *Alcaligenes* sp. telah dugunakan dalam penurunan bahan buangan bulu. Dalam media basal dengan dan tanpa logam berat, bakteria hanya menurunkan 1 g/L bulu dan menghasilkan 8.84 U/mL keratinase dalam 3 hari. Selepas pengoptimuman faktor-faktor fizikal dan budaya mempengaruhi hasil keratinase menggunakan satu faktor-dalam-satu-masa dan kaedah gerak balas permukaan, Penurunan lengkap 5 g/L bulu dan peningkatan 10 kali ganda dalam pengeluaran keratinase (88.4 U/mL). Bakteria ini telah disekat gerak dalam agen pengelan gellan gum dengan keadaan dioptimumkan. Dengan 0.8% kepekatan gula gellan, manik saiz 3 mm dan 250 beban sel awal, sel-sel sekat gerak telah dinurunkan bulu pada kadar yang lebih cepat dan menghasilkan aktiviti keratinase lebih tinggi dalam media yang dioptimumkan. Kedua-dua sel bebas dan sekat gerak dengan cekap penunrunkan bulu melanised keras lebih cepat daripada bulu bukan melanised. Kedua-duanya menunjukkan keutamaan tinggi untuk bulu hitam dan penurunan lengkap 5 g/L bulu hitam untuk debu dan serat telah dicapai pada 18 dan 24 jam untuk sel-sel bebas dan sekat gerak, masing-masing. Menariknya, strain yang terpilih menurunkan sepenuhnya hitam dalam kelalang yang mengandungi campuran jumlah yang sama bulu hitam, coklat dan putih dengan sisa

bulu rachises coklat pada akhir 36 jam pengeraman. Tambahan pula, dengan media dioptimumkan, sejumlah besar sehingga 50 g/L bulu telah diturunkan oleh sel bebabs AQ05-001 manakala sel-sel sekat gerak gerak menurunkan sehingga 40 g/L dalam 54 dan 60 jam, masing-masing dengan paduan yang sangat berjumlah protein larut hidrolisat yang kaya dengan kedua-dua asid amino penting dan tidak penting seperti leucina, isoleucina, cysteina, asid aspartik dan prolina. Kedua-dua sel bebas dan sekat gerak menunkan bulu sarat dengan tunggal atau kombinasi logam berat dengan pengeluaran serentak enzim keratinase lebih tinggi. Sel-sel yang terkandung diterima sehingga 30 ppm Ag, 20 ppm Co, 15 ppm Cu, 5 ppm Hg dan Pb manakala sel-sel hidup bebas diterima hanya 1 ppm Hg dan Pb. Selain itu, sel-sel dalam gellan gam diturunkan kira-kira 90% daripada bulu dalam media yang mengandungi campuran 5 ppm dari setiap Ag, As, Co, Cu, Ni dan 10 ppm dari setiap Ag, Co dan Cu. Manik yang sama telah digunakan dalam beberapa kitaran penurunan bulu tanpa penerapan asid mineral. Manik digunakan semula dalam biopenurunan bulu tercemar dengan logam berat yang berbeza dalam penapaian separuh berterusan sehingga tujuh kitaran. Keratinase mentah yang dihasilkan oleh strain AQ05-001 memaparkan aktiviti maksimum ke atas suhu antara 30-60 ℃, pH 7-9 dan sangat direncatkan oleh perencat serina, Tween20, Trixton-100 dan natrium dodesil sulfat. Walau bagaimanapun, sel keratinase mentah bebas tidak dapat mernurunkan bulu, tetapi penurunan hanya beberapa bulu di hadapan agen penurunan. Selain keratinase tinggi dan hasil protein larut, strain ini boleh digunakan dalam pengurusan semua sisa bulu, pengeluaran protein hidrolisat kaya untuk makanan haiwan dan juga dalam pengeluaran perindustrian enzim berharga keratinase dengan kecekapan yang tinggi dalam penununurunan kedua-dua sangat sukar melanised dan kurang bulu bukan melanised. Tambahan pula, bukan sahaja boleh diterima logam berat yang boleh dimanfaatkan dalam biopemulihan sisa logam berat tercemar bulu, tetapi ia juga boleh menggunakan sisa tercemar sebagai substrat untuk pengeluaran keratinase.

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Ibrahim Yusuf,
20-May-2016

I certify that a Thesis Examination Committee has met on 13 May 2016 to conduct the final examination of Ibrahim Yusuf on his thesis entitled "Biodegradation of Recalcitrant Chicken Feather Wastes by Locally Isolated Heavy Metal Tolerant *Alcaligenes* sp. Strain AQ05-001" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Umi Kalsom binti Md Shah, PhD
Associated Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha bt Raja Abd. Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Janna Ong binti Abdullah, PhD
Associated Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Hiroyuki Futamata, PhD
Professor
Shizuoka University
Japan
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 July 2016

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Siti Aqlima Ahmad, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia
(Chairman)

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

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

Phang Lai Yee, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
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of Supervisory Committee: Dr. Siti Aqlima Ahmad

Signature: _____
Name of Member
of Supervisory Committee: Associate Professor Dr. Mohd Yunus Abd Shukor

Signature: _____
Name of Member
of Supervisory Committee: Professor Dr. Mohd Arif Syed

Signature: _____
Name of Member
of Supervisory Committee: Professor Dr. Phang Lai Yee

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LIST OF ABBREVIATIONS

CFU	Colony forming units
DTNB	Dithiobis (2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
FBA	Feather basal agar
FBB	Feather basal broth
FD	Feather degradation
FDB	Feather degrading bacteria
FDM	Feather degrading micro-organisms
g	Gram
ICL	Initial cell load
KA	Keratinase activities
kDa	Kilo Daltons
L	Litre
mL	Millilitre
MM	Molecular mass
NCBI	National centre for biotechnology information
OFAT	One factor at a time
PBFD	Placket-Burman factorial design
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
SMA	Skim milk agar
SmF/LF	Submerged or Liquid Fermentation
SSF	Solid state fermentation

TCA	Trichloroacetic acid
TSE	Transmissible spongiform encephalitis
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

Chicken continue to form an important part of human meal as cheap source of protein. In the local or industrial processing of these chicken, large quantity of different types of feather are produced as waste (Korniłłowicz-Kowalska and Bohacz, 2011). These feather wastes which are made up of fibrous, insoluble, rigid structural keratins, are generally resistant to bacterial and enzymatic degradation (Anbu *et al*., 2005). They are often disposed traditionally through processes such as burning, chemical treatments and land fillings, the methods that are not environmentally sustainable and also result in the destruction of some essential amino acids (Farag and Hassan, 2004; Rajput and Gupta, 2013). However, in order to dispose the feather wastes effectively and to harness their potentials for wealth, the need for an alternative safer method using microorganisms is highly necessary and is receiving attention from biotechnologists and related professions in the last few decades.

A number of feather degrading microorganisms (FDMs) with varying inherent capability to degrade feather and produce keratinase have been isolated and reported from different environmental sources (Anbu *et al*., 2008; Desai *et al*., 2010; Tatineni *et al*., 2008). The growth of these FDMs and their ability to effectively produce keratinase and degrade feathers are grossly influenced by various physical and nutritional factors. Optimisation of these factors is therefore important in order to maximise their potential use. One-factor-at-a-time (OFAT) optimisation technique is often used to achieved optimum keratinase production (Harde *et al*., 2011), but the method has some limitations among which is lack of capability to address the interactive effects between different factors (Dutta *et al*., 2004; Harde *et al.*, 2011; Okoroma *et al*., 2012; Ramnani and Gupta, 2004). Statistical optimisation approaches are used to correct the limitations of the OFATs and have been used successfully in optimisation of many biological processes (Fakhfakh-zouari *et al*., 2010).

Similarly, the continued use of chicken feathers as an economical and easy biosorbent material to remove heavy metals from surface water and effluents from industries has also resulted in generation of feather-laden heavy metals (secondary feather waste) which are also recalcitrant to degradation due to the toxic nature of heavy metals to microorganisms and also as the inhibitors of enzymatic activities (Cabrera *et al.*, 2006) thereby compounding biodegradation process. Due to tough and toxic nature of these feather wastes, there is a need for an alternative search for FDM that could withstand high concentrations of toxic metals and utilise the toxic wastes as a substrate to produce keratinase enzymes. The kind of such microorganism will be very important in the management of tonnes of secondary chemically polluted feather wastes and production of industrially important keratinase enzyme.

In the recent decades, white chicken feather wastes have become the major substrate of degradation in many studies that reported biodegradation of chicken feathers despite the

availability of other pigmented feathers (Gessesse *et al*., 2003; Sahoo *et al*., 2012). However, due to recent evidences which suggest that pigmented feathers are more harder to degrade by FDMs because of the presence of melanin pigments (Goldstein *et al*., 2004; Gunderson and Frame, 2008; McGraw, 2006), melanised chicken feathers are not fully utilised to produce valuable products such as amino acids and keratinase and are still being disposed in traditional ways.

The use of immobilised cells as a microbial biocatalyst has gained attention over free cells due to higher efficiency of catalysis, operational stability, high cell density retention and reusability (Prakash *et al*., 2010b). Production of keratinase and degradation of feather using free cells of *Bacillus*, fungi and *Streptomyces* in shake flasks using carrier such as alginate, k-carrageenan, agar and chitosan has been described. However, there is no report on the use of gellan gum as a carrier for the immobilisation of feather-degrading bacteria to produce keratinase.

In the view of the above, the study aim to isolate a native bacterium of Malaysia with a potential of withstanding toxicity of heavy metals and ability to degrade melanised feathers.

Thus, the objectives of this study are:

1. To isolate, identify and select heavy metal tolerant feather-degrading bacterium.
2. To optimise physical and nutritional conditions of the bacterium for maximum growth, keratinase production and feather degradation.
3. To immobilise the bacterium, optimise the immobilisation conditions and investigate degradation ability of the immobilised bacterial cells on melanised, non-melanised and mixed coloured feathers in comparison with free cells.
4. To determine the ability of free and immobilised bacterial cells to produce keratinase and degrade heavy metal polluted feather in batch and semi-continuous cultivation.
5. To characterise crude keratinase produced from free and immobilised cells and to determine their feather-degrading ability with or without reducing agents.
6. To analyse amino acid contents of hydrolysates produced from melanised and non-melanised by free cells of *Alcaligenes* sp. AQ05-001 at certain intervals during cultivation.

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