

PRODUCTION OF CYSTEINE AND METHIONINE BY SELECTED
PROTEOLYTIC BACTERIA FROM CHICKEN SLURRY

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Philosophy

Faculty of Science
Universiti Teknologi Malaysia

JULY 2019

DEDICATION

Specially dedicated to my dearest family and match-made-in-heaven of mine,
who had being my pillar of strength since Day 1

ACKNOWLEDGEMENT

I would like to express my appreciation to my supervisors, Dr Adibah Yahya and AP Dr Madihah Md Salleh for their care, guidance, and moral support. An utmost appreciation to research grant AS129 that had been the major financial source to ensure the completion of data collection presented in this thesis.

Much obliged to my family especially my dearest mom; Mrs Sarah Bujang and dad; Mr Haron Narashid Abd Rashid for their untiring love, ceaseless support and encouragement. My special appreciation to Mr Goh Sing Leng; Managing Director of Ayam Kempas Sdn Bhd for his generosity in supplying the raw materials for this research, Mrs Sawiyah Tori from UKM who had share her knowledge and gave me her extensive guidance throughout the completion of the formic acid analysis and Mrs Siti Noreen Kasim from VNK Supply & Services for helping me in purchasing the chemicals and lab ware consumables. Not forgetting my best friend; Farah Syazwani Abdul Ghafar and lab mates; Muhammad Afiq Khir Anuar, Nor Syahiran Zahidin, Mohamed Roslan Mohamad Ikubar, and Rohaya Mohd Noor who had been sharing ideas and bringing smiles to my face during the lowest points in my pursuit for knowledge. I would also like to thank the ICL 1 Lab assistant; Mr Muhammad Hanif Muhammad Hatta and those who had given their assistance and support in any way. Next, my sincere courtesy to both Faculty of Science for the research facilities and PSZ for the endless supply of research materials.

Last but not least, a special gratitude to my match-made-in-heaven; Amir Izzat Mahfar for the pushing factor he provided which inspired me to go on thriving; without which it would have been difficult.

ABSTRACT

The continuous growth of poultry industry in Malaysia has resulted in a significant increase of waste by products generation that could be potentially used as renewable resource for the production of value added products. However, these wastes were not efficiently utilized due to the lack of innovation and scientific research. Thus, this research focused on utilizing the indigenous proteolytic bacteria isolated from chicken viscera slurry to produce cysteine and methionine as supplements in cat food production. Twenty-nine pure bacteria strains were isolated where only three potential proteolytic strains coded as H1, L4 and L6 were selected for further investigation based on their halo zone formation and protease activities. Seven different microbial co-cultures (H1, L4, L6, H1L4, H1L6, L4L6 and H1L4L6) were tested at three different temperatures (37 °C, 45 °C and 56 °C). The initial pH and total fermentation time remained constant in order to optimize protein production. The highest protein concentration was observed in product incorporated with co-culture H1L6 (12.025 mg/mL). Amino acid analysis was carried out to determine whether those with the highest protein concentration also produced the highest amount of cysteine and methionine which served as the essential precursors of the chicken flavour. Product from co-cultures H1L6 produced significant amount of methionine at 56 °C which is 29.130 mg/mL whereby the highest production of cysteine (1.760 mg/mL) is seen in the product incorporated with L6 alone at 56 °C as well. In conclusion, the best incorporated co-cultures to yield the highest amount of cysteine and methionine are culture L6 and co-cultures H1L6 respectively and the optimum temperature is at 56 °C. The end products containing both cysteine and methionine can later be added in pet food particularly for cats to further test their palatability. With thorough optimization, this research will highly be beneficial to the rapidly-growing pet food industry in Malaysia.

ABSTRAK

Kemajuan industri ternakan di Malaysia telah menyebabkan peningkatan pengeluaran sisa buangan yang berpotensi untuk digunakan sebagai sumber yang boleh diperbaharui dan seterusnya menghasilkan produk dengan nilai tambah. Walau bagaimanapun, sisa-sisa ini tidak digunakan secara efisien kerana kekurangan penyelidikan saintifik dan inovasi. Oleh itu, penyelidikan ini bertujuan untuk menggunakan bakteria proteolitik yang dipencilkan dari dalam sisa perut ayam untuk menghasilkan sisteina dan metionina sebagai bahan tambah dalam penghasilan makanan kucing. Dua puluh sembilan bakteria telah dipencilkan dan hanya tiga bakteria proteolitik telah dipilih sebagai bakteria yang berpotensi untuk digunakan dalam penapaian iaitu bakteria H1, L4 dan L6 berdasarkan pembentukan zon lingkaran dan aktiviti enzim protease. Untuk penghasilan protein yang optimum, tujuh kombinasi bakteria (H1, L4, L6, H1L4, H1L6, L4L6 dan H1L4L6) telah diuji pada 3 suhu berbeza (37 °C, 45 °C dan 56 °C). Manakala, pH awal dan jangka masa penapaian adalah tetap. Kepekatan protein tertinggi diperhatikan dalam produk yang dibantu oleh gabungan bakteria H1L6 (12.025 mg/mL). Analisis asid amino telah dijalankan untuk menentukan sama ada kepekatan sisteina dan metionina dalam protein yang dihasilkan adalah tinggi, memandangkan mereka merupakan prekursor utama kepada perasa ayam. Produk dari gabungan bakteria H1L6 menghasilkan jumlah metionina yang sangat tinggi pada suhu 56 °C iaitu 29.130 mg/mL. Pengeluaran sisteina tertinggi (1.760 mg/mL) pula dapat dilihat dalam produk yang dibantu oleh bakteria L6 juga pada suhu 56°C. Kesimpulannya, gabungan bakteria terbaik bagi penghasilan sisteina dan metionina adalah bakteria L6 dan gabungan H1L6 dan suhu optimum adalah pada 56 °C. Produk akhir yang mengandungi sisteina dan metionina boleh ditambah ke dalam makanan haiwan kesayangan terutama untuk kucing bagi menguji kesesuaiannya. Dengan pengoptimuman menyeluruh, penyelidikan ini akan memberi manfaat kepada industri makanan haiwan kesayangan yang sedang berkembang pesat di Malaysia.

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LIST OF ABBREVIATIONS

16S rRNA	-	16S ribosomal RNA
AAFCO	-	Association of American Feed Control Officials
ADMI	-	American Dye Manufacturer's Institute
As	-	Arsenic
BLAST	-	Basic Local Alignment Search Tool
BSE	-	Bovine Spongiform Encephalopathy
C : N	-	Carbon : Nitrogen
CCA	-	Calcium Caseinate Agar
Cd	-	Cadmium
GA	-	Genetic Algorithm
PSO	-	Particle Swarm Optimization
cfu/g	-	Colony forming unit per gram
cP	-	Centipoise
Cys	-	Cysteine
DCM	-	dilated cardiomyopathy
EC	-	European Commission
EU	-	European Union
FDA	-	Food and Drug Administration
g	-	Gram
GDP	-	Gross Domestic Product
HBr	-	Hydrogen bromide
HCl	-	Hydrogen chloride
Hg	-	Mercury
kcal/kg	-	kilocalorie per kilogram
kDa	-	kilodaltons
L	-	Liter
Met	-	Methionine
mL	-	Milliliter
mM	-	Millimolar
MPN/g	-	most probable number per gram

N	-	Normality
OD	-	Optical density (absorbance)
Pb	-	Lead
PCR	-	polymerase chain reaction
ppm	-	parts per million
PUFA	-	Polyunsaturated fatty acids
rpm	-	rotation per minute
S.O.P.	-	Standard Operating Procedure
TAE	-	Tris-Acetate-EDTA
TSE	-	Transmissible Spongiform Encephalopathy
v/v	-	Volume per volume
w/w	-	Weight per weight

LIST OF SYMBOLS

°C	-	degree Celcius
%	-	per cent
μL	-	microliter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Concerns over the commercially valuable products produced from microorganisms have been growing vastly in recent years and this is attributed mainly by the escalating global energy and environmental problems. Thus, this current situation has stimulated and triggered the instincts of researchers from all walks of life to develop promising methods for producing almost everything through green-based technology.

Found ubiquitously in nature, proteases are of vital importance for the growth of cell and cell differentiation (Gupta *et al.*, 2002; Queiroga *et al.*, 2012). Given the fact that microorganisms-produced extracellular proteases can be isolated with a facile method from the growth medium, they are sought after commercially with a very high demand (Queiroga *et al.*, 2013). The protease compasses myriads of industrial scale applications such as being utilized in food and feed industries, synthesis of peptides, and waste management.

Obliged to their biological and economic significance among hydrolytic enzymes, microbial-derived proteases have been substantially studied, yet they remain a trending research topic. Although myriads of proteases isolated from microbial sources are at one's fingertip, the industries favoured those that can release significant amounts of extracellular enzymes (Queiroga *et al.*, 2013). Besides, although many review papers are available on microbial alkaline protease producers, yet reports and reviews on microbial acidic proteases can hardly be found. Based on works done by Jamdar and Harikumar in 2005, they reported that chicken intestine is an excellent source of acid proteases.

Hence, this research effort focused on the isolation and selection of protein-degrading bacteria exhibiting proteolytic activity from chicken viscera – mainly intestines, hearts, and livers. Selected proteolytic bacteria exhibiting high protein productions were used for the production of cysteine and methionine which are important amino acids as precursor for taurine biosynthesis. Through fermentation of chicken viscera slurry, a fermented cocktail containing both amino acid could potentially be used as supplement in cat food production.

1.2 Problem Statement

The production and consumption of poultry products have been on the increase globally (Lasekan *et al.*, 2013). Provided that, the waste production in terms of the animal by-products also proliferate. In Malaysia specifically, there are numerous slaughtering farms for chicken which produced tonnes of by-products daily. Poor management of the abundant by-products is at an alarming rate. For instance, in aquaculture industry, the chicken by-products are normally served as feeds where with improper storage, they will start to decompose later causing myriads of problems including odour problem, water pollution, and terrible cleanliness issue.

Most of the time these by-products are only considered as waste and resulting in under-utilization of their potential especially in the pet food industry. Pet food industry is one of those vast-growing industries, which have been performing well despite of economic downturn. Escalating number of pet owners who treat their pets like family members and demand for natural and high quality treats have pushed manufacturers to introduce high- priced and value-added premium products that contain specialized ingredients. (Intelligence & Partner, 2015). Cats for instance, are known for their inability to synthesise taurine thus to cater their needs, the palatants for cat food need to have taurine as an essential ingredient. Cysteine and methionine are amino acids with sulphur-based, serving as the vital precursors for taurine synthesis (Markwell & Earle, 1995). These amino acids also serve as flavour enhancer.

Due to the aforementioned problems, this research proposed a much better option for value-added utilization of poultry waste by aiming to produce protein consisting of both cysteine and methionine to be later used as palatant in cat food by harnessing the chicken viscera waste and their indigenous microflora content focusing on those having proteolytic properties.

1.3 Research Objectives

The objectives of this research were :

1. to analyse the microelements presence in chicken viscera and selecting suitable parts of the chicken viscera to be used for protein hydrolysate production containing cysteine & methionine.
2. to isolate, select, and phylogenetically characterize the potential proteolytic bacteria from the chicken viscera.
3. to produce cysteine and methionine through fermentation of selected part of chicken viscera as substrate harnessing the selected proteolytic bacteria.

1.4 Scope of Research

This research focused on utilizing the indigenous microorganisms specifically protein-degrading bacteria (proteolytes) from the chicken viscera to produce protein hydrolysate consisting two amino acids namely cysteine and methionine. Firstly, the chicken viscera (intestines, livers, and hearts) was thoroughly analysed before selecting the suitable part to be used as substrate for fermentation process. Isolation, screening for selection, and phylogenetical characterization of potential proteolytes from the chicken viscera were also carried out. Next, different microbial cocultures

were form from the selected proteolytes in order to optimize the protein hydrolysate production containing cysteine and methionine namely (i) H1, (ii) L4, (iii) L6, (iv) H1L4, (v) H1L6, (vi) L4L6, and (vii) H1L4L6. Temperatures used were also varied at (i) 37 °C, (ii) 45 °C, & (iii) 55 °C whereas the initial pH and fermentation time are fixed at pH 2.8 (Jamdar & Harikumar, 2008a) and 12 hours respectively. Finally, samples with the highest protein concentration which were determined by Lowry assay underwent performic acid analysis to quantify the amount of cysteine and methione produced.

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