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AUTOCLAVE-CHEMICAL HYDROLYSIS OF CHICKEN FEATHER FOR PROTEIN HYDROLYSATE PRODUCTION

CHEONG CHOOI WEI

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By

CHEONG CHOOI WEI

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AUTOCLAVE-CHEMICAL HYDROLYSIS OF CHICKEN FEATHER FOR PROTEIN HYDROLYSATE PRODUCTION

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April 2018

Chairman: Phang Lai Yee, PhD
Faculty: Biotechnology and Biomolecular Sciences

Millions tonnes of feather waste are generated every year. Various treatments on feather waste have been developed in order to add value to them. However, these methods have their pros and cons. Autoclave-chemical treatment could hydrolyse feather within a short period but harsh conditions could lead to excessive destruction on protein and amino acids. Biological treatment is an eco-friendly treatment which could hydrolyse feather with minimum protein and amino acid destruction but it required a long reaction time. Therefore, treatment modification is necessary in order to enhance the feather hydrolysis and produce feather hydrolysate consists high amount of protein and amino acids. The objectives of this study were to investigate the effect of autoclave-chemical treatment on chicken feather hydrolysis and to examine the effect of autoclave-alkaline as pretreatment on enzymatic hydrolysis of chicken feather. Sodium hydroxide (NaOH) was selected based on the screening result and the temperature was fixed at 105°C. The NaOH concentration and autoclave holding time ranged from 0.01 M to 0.10 M and 1 min to 10 min, respectively were used in the subsequent experiments. The effect of autoclave-alkaline treatment (AAT) on feather was investigated using response surface methodology (RSM). NaOH concentration and holding time were the significant parameters in AAT that affect the feather hydrolysis and protein production ($p < 0.05$). The ideal conditions for AAT were 0.08 M of NaOH, 7 min holding time at 105°C in which 85.59% of feather were solubilized and 0.75 g/g of soluble protein with 633.50 mg/g of free amino acid could be recovered from chicken feather. Autoclave-alkaline method was also proposed to be used as pretreatment in this study in order to enhance the performance of the subsequent biological treatment. The effect of autoclave-alkaline as pretreatment on the enzymatic hydrolysis of chicken feather with Savinase® Ultra 16L by RSM was investigated. NaOH concentration was significant parameter that affected the subsequent enzymatic reaction on feather hydrolysis and protein production ($p < 0.05$). The pretreated chicken feather with optimized autoclave-alkaline (0.07 M of NaOH, 2 min holding time at 105°C) could improve Savinase hydrolysis up to 14 times with 80.81% of feather hydrolysis and recovered 0.69 g/g of soluble protein as well as 673.80 mg/g of free

amino acid. Therefore, AAT is a potential method for feathers solubilization, as well as, recovery of soluble protein and free amino acid from chicken feather waste. Autoclave-alkaline pretreatment also could enhance enzyme reaction to degrade chicken feather to a great extent and generate feather hydrolysates rich in amino acids.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

HIDROLISIS AUTOKLAF-KIMIA BULU AYAM UNTUK PENGHASILAN PROTEIN HIDROLISAT

Oleh

CHEONG CHOOI WEI

April 2018

Pengerusi: Phang Lai Yee, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Berjuta-juta tan bulu ayam telah dihasilkan setiap tahun sebagai sisa buangan. Pelbagai rawatan terhadap sisa bulu telah ditemui untuk menambah nilai sisa bulu tersebut. Walau bagaimanapun, rawatan-rawatan tersebut mempunyai kebaikan dan keburukan. Rawatan kimia-autoklaf mampu menghidrolisis bulu ayam dalam tempoh yang singkat tetapi keadaan rawatan yang tidak bersesuaian boleh mengakibatkan pemusnahan berlebihan terhadap protein dan asid amino. Rawatan biologi adalah rawatan mesra alam yang boleh menghidrolisis bulu dengan mengurangkan pemusnahan protein dan asid amino tetapi memerlukan masa reaksi yang panjang. Oleh itu, pengubahsuaian rawatan diperlukan untuk mempertingkatkan hidrolisis bulu dan menghasilkan hidrolisat bulu yang mengandungi jumlah protein dan asid amino yang tinggi. Objektif kajian ini adalah untuk mengkaji kesan rawatan kimia-autoklaf terhadap hidrolisis bulu ayam dan juga untuk mengkaji kesan pra-rawatan autoklaf-alkali terhadap hidrolisis enzimatik bulu ayam. Berdasarkan keputusan penyaringan, natrium hidroksida (NaOH) telah dipilih dan suhu telah ditetapkan pada 105°C. Kepekatan NaOH dan masa pegangan dalam autoklaf masing-masing adalah antara 0.01 M hingga 0.10 M dan 1 minit hingga 10 minit. Kesan rawatan autoklaf-alkali (AAT) terhadap bulu telah dikajikan dengan menggunakan kaedah gerak balas permukaan (RSM). Kepekatan NaOH dan masa pegangan adalah parameter penting dalam AAT yang mempengaruhi hidrolisis bulu dan penghasilan protein ($p < 0.05$). Keadaan optima untuk AAT adalah 0.08 M NaOH, dengan masa pegangan selama 7 minit dan suhu 105°C, di mana 85.59% bulu telah dilarutkan dan 0.75 g/g hasil protein dengan 633.50 mg/g asid amino bebas boleh dipulih daripada bulu ayam. Kaedah autoklaf-alkali juga dicadangkan untuk digunakan sebagai pra-rawatan untuk meningkatkan prestasi rawatan biologi berikutnya. Kesan pra-rawatan autoklaf-alkali terhadap rawatan enzim yang berikutnya telah dikajikan dengan menggunakan Savinase® Ultra 16 L dan kaedah RSM. Kepekatan NaOH adalah parameter penting yang mempengaruhi tindak

balas enzimatik berikutnya pada hidrolisis bulu dan pengeluaran protein ($p < 0.05$). Bulu ayam yang telah dirawat dengan pra-rawatan autoklaf-alkali dalam keadaan optima (0.07 M NaOH, 2 minit masa pegangan pada 105°C) mampu meningkatkan hidrolisis Savinase sehingga 14 kali ganda dengan 80.81% hidrolisis bulu dan 0.69 g/g protein serta 673.80 mg/g asid amino bebas dipulih. Oleh itu, AAT adalah kaedah yang berpotensi untuk melarutkan bulu ayam dan memulihkan protein dengan asid amino bebas daripada bulu ayam. Pra-rawatan autoklaf-alkali juga boleh mempertingkatkan reaksi enzim untuk menguraikan bulu ayam dan menghasilkan hidrolisat bulu yang kaya dengan asid amino.

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I certify that a Thesis Examination Committee has met on 26 April 2018 to conduct the final examination of Cheong Chooi Wei on her thesis entitled "Autoclave-Chemical Hydrolysis of Chicken Feather for Protein Hydrolysate Production" 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

Noorjahan Banu binti Mohammed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Normala bt Halimoon, PhD
Senior Lecturer
Faculty of Environmental Studies
Universiti Putra Malaysia
(Internal Examiner)

Fauziah binti Shahul Hamid, PhD
Senior Lecturer
University of Malaya
Malaysia
(External Examiner)

RUSLI HAJI ABDULLAH, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 30 July 2018

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

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

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

Ooi Peck Toung, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

____
ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name and Matric No.: Cheong Chooi Wei, GS43523

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Signature : ____________________
Name of chairman of supervisory committee : Phang Lai Yee

Signature : ____________________
Name of member of supervisory committee : Ooi Peck Toung

Signature : ____________________
Name of member of supervisory committee : Siti Aqlima Ahmad

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LIST OF ABBREVIATIONS

AAS	Autoclave-alkaline Savinase combined treatment
AAT	Autoclave-alkaline treatment
°C	Degree Celsius
%	Percentage
A_{600nm}	Optical density at wavelength 600 nanometer
µL	Microliter
µm	Micrometer
$CaCl_2$	Calcium chloride
g	Gram
L	Litre
M	Molar
MT	Million tonnes
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
RSM	Response surface methodology
CCD	Central composite design
FTIR	Fourier transform infrared
CHNS	Carbon, hydrogen, nitrogen and sulphur
USA	United State of America
IEC	Ion exchange chromatography
Psi	Pounds per square inch
GLN	Glutamine
GLU	Glutamic acid
ASP	Aspartic acid
ASN	Asparagine
NH_4OH	Ammonium Hydroxide
NaCN	Sodium cyanide
DTT	Dithiothreitol

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nowadays, the rate of consumption poultry meat is increasing year by year. In Malaysia, poultry meat consumption has reached 1632 million tonnes in 2017 (United State Department of Agriculture, 2017). This high consumption rate on poutry meat is probably due to its cheap price among the protein sources and it is suitable for all ethic and culture groups. The sharp demand on poultry meat leads to the production of noticeable amounts of by-products such as chicken feathers, from poultry processing plants. Since, chicken feathers constitute 10% of total chicken weight (Thyagarajan *et al*., 2013), million tonnes of chicken feathers are generated annually in the worldwide poultry industries as the consumption of poultry meat increases (Ali *et al*., 2011). Some of these feather wastes are utilized to produce dusters or decorative products as well as high-class bedding. However, most of the feathers are disposed in dumps, landfills and/or incinerators. These methods may cause contamination to our environment by generating greenhouse gases such as methane and carbon dioxide (Acda, 2010b). The application of these disposal methods were also limited due to lack of landfill spaces (Acda, 2010b) and high expenses (Tonkova *et al.*, 2009).

Feather is made up of more than 90% of protein, namely keratin (Mokrejs *et al.*, 2011) and it also contains high levels of certain amino acid which are cysteine, glycine, arginine, and phenylalanine (Kumar *et al*., 2012). Nowadays, essential amino acids were highly demanded in global market as it is expected to reach 10.1 million ton which is valued at USD 35.4 billion by 2022 (Radiant Insight Inc., 2015). Instead of becoming environmental pollutant, the chicken feathers can be transformed into valuable and marketable products. Up to date, they could be used to make feather meal (Kumar *et al*., 2012), bio-fertilizers (Saber *et al.*, 2010) and thermoplastic (Ullah *et al*., 2011). Lately, feather was also suggested to be used as biomedical reagent for wound healing (Wang *et al*., 2017). However, feather is difficult to be hydrolyzed or degraded due to its high constitution of keratins (Mokrejs *et al.*, 2011). Keratin consists of large amount of cysteine containing sulphur which is the building block of the disulphide bridge. Keratin is tightly packed either in α-helix or β-sheets into the keratin supercoiled polypeptides. These polypeptides are further stabilized by hydrogen bond and hydrophobic interaction (Brandelli, 2008). Also, keratin is the protein that has the high mechanical and temperature stability (Brandelli, 2008).

There are various treatments reported to be able to treat feather waste in order to add value to them (Barone and Schmidt, 2006; Chinta *et al*., 2013; Lakshmi *et al*., 2013; Mokrejs *et al.*, 2011b; Paul *et al.*, 2013; Staroń *et al.*, 2014; Stiborova *et al.*, 2016).

Generally, they are categorized into three groups namely, physical, chemical and biological treatments. Each of these treatments has the pros and cons in feather degradation, as well as, protein and amino acid recovering (Cheong *et al*., 2017). Physical treatment involves fast reaction and the treatment can be easily handled. However, the feathers are usually degraded at high temperature or/and pressure which may lead to excess denaturation of certain amino acids and high amount of energy is needed for the machine operation in physical treatment. Chemical treatment can be performed easily by applying strong acids or alkaline to degrade the feathers. The use of reducing agents such as 2-mercaptoethanol, dithiothreitol (DTT), sodium m-bisulphite and sodium bisulphite, also could effectively degrade chicken feathers into soluble forms (Sinkiewicz *et al*., 2017). However, chemical treatment may also lead to loss of certain essential amino acid. For example, tryptophan, cystine, serine, and threonine were destroyed in acid hydrolysis (Haurowitz, 1955).

Biological treatment is an environmental friendly method which involves the utilization of keratinase producing microorganisms or keratinase alone to break down the rigid bonds in feathers. However, this method is time consuming due to the low reaction rate of the keratinase producing microorganisms or keratinase on feather hydrolysis (Kani *et al*., 2012; Kim *et al.*, 2005; Matikeviciene *et al*., 2009; Poovendran *et al*., 2011). Considering the pros and cons of each treatment, it is necessary to develop an effective method for feathers hydrolysis in order to recover high amount of good quality protein and/or amino acid. Recently, studies of feather degradation by using combination of physical, chemical and biological treatments were done either in single step or two-steps. The combined treatments could diminish the disadvantages of the treatment methods while retaining the quality of recovered soluble protein and amino acids. For example, microwave-alkaline pretreatment (450 W, 0.05 M of NaOH for 10 min) has enhance the subsequent enzymatic hydrolysis of chicken feather to about 88% degradation with about 94% of soluble protein was recovered (Lee, 2016).

Autoclave is a thermal method that can be easily applied. Studies show that it might hydrolyse protein materials at appropriate conditions (Badadani *et al*., 2007; Eddie *et al*., 2016; Moore and Stem, 1963). Amino acids like glutamine and asparagine in the feather were destroyed under high-temperature and high pressure conditions (Wu, 2013). According to Taira (1973), 1.1 g/16g N of arginine and 2.0 g/16g N of lysine in soybean was lost after a treatment of 120°C for 30 min and 160°C for 10 min, respectively. However, there was no changes in amino acid when the soybean was treated under 100°C for 5 min (Taira, 1973). Hence, in order to avoid amino acids destruction, a lower temperature setting in the autoclave (105°C, 3 Psi pressure) was proposed in this research.

Strong alkaline may lead to losses of some essential amino acids. Stiborova *et al.* (2016) reported that 1% (w/w) of amino acids could be recovered from the feather being treated with 0.107 M of KOH within 24 hours at 70 °C. This indicated that at mild conditions (mild alkaline concentration and heating) feather could be hydrolyzed to release small amount of amino acids. It could be hypothesized that autoclave-chemical treatment at appropriate conditions may break down the rigid bonds in feathers and hence improve the protein and amino acid recovery. On the other hands, at certain circumstances, autoclave-chemical method may be applied as pretreatment to enhance the hydrolysis performace of biological treatment. Łaba and Szczekała (2013) reported that autoclaving in 10 mM sodium sulfite could enhance the subsequent feather hydrolysis by crude keratinase extracts of *Bacillus cereus* B5esz (86.3% degradation), as well as, the production of amino acids such as leucine, valine, glutamate, glycine, serine and cysteine.

1.2 Problem Statement

Autoclave heating is a common method used in biomass treatment. It has been classically applied for protein hydrolysis by treating the protein or peptide substances with the addition of 6 M hydrochloric acid, at 110°C for 18–24 hours. However, there is no study on the usage of autoclave-alkaline treatment on keratinous materials. Moreover, some studies also claimed that thermal-chemical process affect amino acids yield at high temperature and high concentration of chemicals (Barone and Schmidt, 2006; Guillermo *et al.*, 2016; Mokrejs *et al.*, 2011; Mehta *et al.*, 2014). Hence, research is needed to improve protein and amino acids recovery by thermal-chemical process. It is hypothesized that the overall protein hydrolysis could be improved by thermal-chemical treatment conducted at milder conditions, i.e. lower chemical concentration and temperature.

Biological treatment on chicken feather hydrolysis is an environment friendly method which can produce not only hydrolysates containing soluble proteins but also reduce loss in essential amino acids. However, biological treatment has low reaction rate and low yield, hence these drawbacks limited its application in industrial scale. Pretreatment is a common step in a fermentation process, especially if the biomass is used as feedstock due to its robust structure (Farid *et al*., 2014; Łaba *et al*., 2015; Wanitwattanarumlug *et al*., 2012; Zakaria *et al*., 2015). So, recently combined methods has been proposed in order to reduce the treatment time and to improve the yield of biological treatment. However, the reports on the usage of autoclave to pretreat feather was limited and current developed combined methods were effective in feather degradation but poor in protein recovery (Łaba and Szczekała, 2013; Lee, 2016). Research on treatment modification is needed to enhance solubilization of the feather and improve the recovery of protein and amino acids.

1.3 Research Objectives

General objective:
This study aimed to determine the feasibility of autoclave-chemical method as a treatment and/or pretreatment for chicken feather hydrolysate production.

The specific objectives of this study were:

i. To investigate the effect of autoclave-chemical treatment on chicken feather hydrolysis;
ii. To examine the effect of autoclave-alkaline as pretreatment on enzymatic hydrolysis of chicken feather.

1.4 Research Questions

There are some research questions were underlying with the objectives. The specific research questions of this study were:

i. How much protein could be recovered from chicken feather by autoclave-alkaline treatment?
ii. Did autoclave-alkaline pretreatment enhance the enzymatic hydrolysis of chicken feather?
iii.

1.5 Scope of Study

This study involves three parts which are screening process (Part 1), autoclave-chemical hydrolysis (Part 2) and autoclave-chemical-enzymatic hydrolysis (Part 3). In the screening process, three parameters namely, chemical types, chemical concentration, and holding time involved in autoclave-chemical hydrolysis were examined by using one-factor-at-a-time (OFAT) approach. Autoclave-alkaline as treatment and pretreatment were optimized with Response Surface Methodology (RSM). RSM is as efficient optimization tool which able to explain the effects of multiple factors and their interactions on the responses with reduced number of expriments (Bezerra *et al*., 2008). Center composite design (CCD) was used for the autoclave-alkaline treatment optimization. The effect of NaOH and holding time were the independent variables on total protein and percentage of solubilization were investigated. Savinase, a commercial enzyme was used in the enzymatic hydrolysis (Part 3 of the study). The solubilization of feathers in these treatments were determined based on the sample weight loss. The soluble protein production after the treatments were also analysed. Amino acid profiles of the hydrolysate produced after autoclave-alkaline treatment and autoclave-alkaline-enzymatic treatment were determined. The structural and elemental changes of the treated samples were analyzed by Fourier Transform Infrared (FT-IR) Spectroscopy and Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) analysis.

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