EFFECT OF PH AND ENZYME LOADING ON THE PROTEIN CONCENTRATION DURING COLLOCALIA FUCIPHAGA HYDROLYSIS

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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and from my point of view, this thesis fulfills in terms of scope and quality for the award of Degree of Bachelor Chemical Engineering in Biotechnology.

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I declare that this report is my own work except for the quotations and summaries which have been duly acknowledged.

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I dedicate this entire work to Allah S.W.T because of His blessing and to my family especially to my beloved parents (Mr. Ibrahim Bin Che ‘Ali and Mrs. Wan Munah Bin Abdul Rahman) whose patient and support have facilitated my study, and made my life enjoyable. I also dedicate this work to my supervisor and co-supervisor (Dr. Mimi Sakinah Binti Abdul Muna’im) for their patience in guiding me to complete the work and thesis.

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ABSTRACT

The purpose of this study is to investigate the effect of pH and enzyme loading on the concentration of protein during enzymatic hydrolysis of *Collocalia Fuciphaga. C. Fuciphaga* is refers to the bird nest from one swiftlet species that is used as the sample known to be highly nutritious. Bovine Serum Albumin (BSA), a standard protein solution was used as the benchmark for the reference of the protein concentration. A research had been proposed in this study to optimize the production of protein from *C.Fuciphaga.* The effect of changing of the parameters values during the enzymatic hydrolysis was studied and the higher protein concentrations produced were highlighted. The standard protein curve of Bovine Serum Albumin (BSA) is prepared first with several dilutions for protein standard curve. The results from two significant parameters in the experiment was taken and analyzed. The selection of parameters values was taken from the enzymes' optimum condition where the pH value of Alcalase 2.4L is from pH 7 until pH 10 whiles the temperature is fixed at 50°C. Enzyme loading was varies from 1.5% to 3.0% (v/w). The sample was analyzed using UV-Vis Spectrophotometer to determine the concentration of protein in each sample. The optimum condition is found to be at extraction condition of pH 8.5 and enzyme concentration of 3.0% (v/w). Characterization of the sample had been done and validated the protein composition on the extracted sample.

ABSTRACT

Tujuan kajian ini adalah untuk mengkaji kesan pH dan kepekatan enzim pada kepekatan protein semasa hidrolisis enzim *Collocalia Fuciphaga*. *C. Fuciphaga* adalah merujuk kepada sarang burung dari satu spesies burung walit yang digunakan sebagai sampel kerana ia dikenali untuk menjadi sangat berkhasiat. Serum Albumin Bovine (BSA), larutan protein piawai telah digunakan sebagai penanda aras untuk rujukan kepekatan protein. Satu penyelidikan telah dicadangkan dalam kajian ini untuk mengoptimumkan pengeluaran protein dari *C.Fuciphaga*. Kesan perubahan nilai parameter semasa hidrolisis enzim telah dikaji dan kepekatan protein yang lebih tinggi yang dihasilkan telah diketengahkan. lengkung protein standard Bovine Serum Albumin (BSA) disediakan terlebih dahulu dengan pencairan beberapa sebagai lengkung protein piawai. Keputusan dari dua parameter signifikan dalam eksperimen akan diambil dan dianalisis. Pemilihan nilai parameter telah diambil dari keadaan optimum enzim di mana nilai pH Alcalase 2.4L adalah dari pH 7 sehingga pH 10 manakala suhu ditetapkan pada 50°C. Keadaan optimum didapati berada pada keadaan pengekstrakan pH 8.5 dan kepekatan enzim sebanyak 3.0% (v / w). Sampel ini kemudiannya akan dianalisis menggunakan Spectrophotometer UV-Vis untuk menentukan kepekatan protein dalam setiap sampel. Pencirian sampel telah dilakukan dan mengesahkan komposisi protein pada sampel yang diekstrak.

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LIST OF ABBREVIATIONS

EBN	Edible Bird Nest
FTIR	Fourier Transform Infrared Spectroscopy
OFAT	One Factor at a Time
DNS	Dinitrosalicylic acid
BSA	Bovine Serum Albumin
AU	Anson Unit
NaOH	Sodium Hyroxide
OD	Optical Density
rpm	Round per Minute
SEM	Scanning Electron Microscopy

LIST OF SYMBOLS

^{o}C	-	Degree Celsius
%	-	Percent
g	-	Gram
L	-	Liter
rpm	-	Rotation Per Minutes
M	-	Molarity
ml	-	Milliliter
v	-	Vibration wavelength
cm^{-1}	-	Reciprocal Centimeter
mg	-	Milligram
Abs	-	Absorbance
ml	-	Millilitre
min	-	Minute
hr	-	Hour

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Proteins are essential food components because they are a source of amino acids needed for growth and maintenance and provide functional properties to foods. Commercially available protein foods are obtained from a range of animal and plant sources and are used as functional ingredients (Martina. 2002). Due to the increasing costs and limited supplies of animal proteins and since vegetable protein is the most abundant source of protein on the Earth, a number of vegetable proteins such as alfalfa leaf, cottonseed, winged bean, peanut and soya have been investigated for possible incorporation into formulated foods.

Protein molecules tend to unfold and even become fully denatured under unfavoured conditions, such as a high temperature, an acidified condition, a high pressure or even excessive shear. Denatured protein molecules will aggregate and/or

crosslink to form larger clusters and, at high concentrations, will form a three-dimensional solid-like network (or a gel). Therefore, proteins are regarded as one of the main classes of building blocks used in many semi-solid foods for conferring mechanical properties (Linqiang et. al., 2008).

For this particular research, the raw material that will be used is *Collocalia Fuciphaga*. *C. fuciphaga* species is popularly known as the White Nest or House Nest swiftlet. *C. fuciphaga* measures about 12 cm in its entire length and weighs about 15 to 18 gm. In recent years, hormone-like substances such as mitogen and avian epithelial growth factor have been found in *C. fuciphaga* (Jie *et al*., 2009).

Edible bird's nest (EBN) is the nest of the swift and is constructed with salivary glue, which is a cementing substance, and may incorporate other materials such as vegetation or feathers. Although EBN mainly contains carbohydrates, amino acids, and mineral salts, the abundant ingredient is composing of glycoproteins (Wu , Wu et al., 2010). Due to its nutritious and medical properties, EBN has been deemed a precious food tonic in Chinese community ever since the Tang (907 AD) and Sung (960–1279 AD) dynasties. Despite the long history of using EBN for medicinal purpose, there have only been a limited number of scientific reports on the health benefits of EBN.

1.2 PROBLEM STATEMENT

The problem of the research is to find out the most appropriate condition that can optimize the protein yield via the enzymatic hydrolysis process. The important part is to search for the protein sources that can be produced in a great amount so that the nutrients from the food can be obtained by all people who in need. Hence the nutritional food is widespread produced all around the world to get all of the healthy life with all of those nutritional benefits. However, the protein extracted from marine life, sometimes not suitable to consumers with heart diseases and blood pressure.

With the researches that had been conducted nowadays, protein from various sources; from plant or from other animal is being looked out. Besides, the most optimum production of the nutritious food is being focused nowadays to produce an optimum condition for the extraction of the protein. The research regarding the optimum condition of the protein concentration of *Collocalia fuciphaga* has not been done before.

1.3 OBJECTIVES

The main objective of this research is to characterize and optimize protein extraction yield from *C.fuciphaga* using enzymatic hydrolysis method. In this research also have a few specific objectives. The specific objectives are:

- To determine the influence of pH on extraction protein yield.
- To determine the influence of enzyme loading on extraction protein yield.
- To characterize the sample by using Fourier Transform Infrared Spectroscopy (FTIR).

1.4 SCOPES OF THE STUDY

Below are the scopes of the study for the research that will be conducted;

- This research will be focusing on the characterization of *C.fuciphaga*mainly using the Ultra-violet Visible spectrophotometer.
- Study the effect of three parameters, the pH of the solution from pH 7-10, and the enzyme loading ranging from 1.5% to 3.0
- The optimal condition of pH, and enzyme loading during the enzymatic hydrolysis will be determined by using Lowry Method One Factor at a Time (OFAT).

1.5 SIGNIFICANCE OF THE PROPOSED STUDY

Nowadays the application of protease is often an attractive means for obtaining better functional properties of food proteins, without impairing their nutritional value. In order to increase and to improve the protein quality produced from the sources of protein, the extracted protein need to undergo the hydrolysis process with the addition of enzyme with several modifications made (Ng et al., 2012). The process of protein extraction using enzymatic hydrolysis method is proved to produce hydrolysates with well-defined peptide profiles (Palupi et al., 2010).

Hence, the study of the extraction protein from Collocalia Fuciphaga with the optimum condition will be conducted so that the nutrients on the protein in the bird nest will be preserve and produce higher protein yield. Besides people that doubtful with the contents of the nest especially the Muslim and also Vegetarian users will leave the doubt and take the benefits of the bird nest. As the sample taken is not from the marine life or any animal sources, so it does not causes allergic to the consumer.
Hence it is really suitable for all type of people who need it.

CHAPTER 2

LITERATURE REVIEW

2.1 SOURCES OF PROTEIN

Protein is essential for the development of the human life as they are really needed for the growth and maintenance of the body. They are sources of amino acids that responsible for many functional properties that influence to foods. The basic structures of protein are composed of small units. The units are consists of small units which are the amino acids units that are also called the building blocks of protein. Protein is an essential nutrient for human many living things. Protein is contained in every part of your body, the skin, muscles, hair, blood, body organs, eyes, even fingernails and bone. Protein is the most plentiful substance in your body after water and no life can survive without protein (Lauritzen, 1992). There are various sources of protein that can be taken nowadays from a two different type of protein sources.

The sources can be classified as the animal protein and the plant protein. Protein does exist for both animal and plant sources. Different type of animal and plant sources have different amount of protein in it. The determination of protein in various sources had been discovered and continuously in the research to know the protein concentration and the quality of the protein. Hence nowadays, a wide range of protein from animal and plant are being commercialized and are available to be used today to be used as useful ingredient for general and specific uses (Hoffman & Falvo, 2004). Protein is however not a primary source of energy but it can be used as energy where the protein have to be metabolized into the much more simplest form into the amino acid from in order to generate energy. (Hoffman & Falvo, 2004)

Figure 2-1 Example of different Sources of Protein

2.1.1 Plant as a Sources of Protein

The source of protein from plant is being nowadays being the alternatives for the consumption rather than the protein from animal. The protein on plants can be obtained

from a lot of sources. Whole grains and cereals are another source of protein. There are a lot of sources of protein that can be obtained nowadays. Proteins from plants contribute over 65 percent on the worldwide basis on the per capita supply of protein (Young & Pellen, 1994) Oats, maize, rice and wheat are examples of protein sources which the concentration of protein is greater than 7 percent (Young & Pellen, 1994). For the vegetarian sources of protein the sources include the legumes, nuts, seeds and fruits. Some of the vegetarian foods with higher concentration of protein include soybeans, lentils, white beans, pigeon peas, almonds, sunflower seeds, cowpeas, walnuts and pumpkin seeds.

Determination of protein in plant had been made in determining the quality and the digestibility of the protein. The plant consider as incomplete protein because of the protein is not having the all the amino acid that should be consumed in human body. According to Hoffman & Falvo (2004), the protein form plant said to be having less protein from animal protein and are generally lacking single or two amino acids. Vegetable protein is the alternative choices for those who are really want to have a result in a reduction in the intake of the saturated fat and cholesterol.

2.1.2 Animal Source of Protein

Proteins from animal sources is the highest quality food sources that been consume on today’s life. The protein that are taken from protein is has the quality higher than the protein from the plant sources. The protein from animal can be consumed from

a lot of sources such as meat, egg, fish, poultry and milk. These protein sources of food have been consume by almost all of the human population around the world for the consumption of the protein in their everyday diet. Based on the statement made by (Hoffman & Falvo, 2004), the protein from animal has the highest quality rating due to the protein in the animal has all the required characteristics as the protein needed. The protein is said to be in the completeness for the animal protein. Also for the benefits taken from total protein consumption, a lot of elderly people had also been benefited from consuming animal sources of protein. Meat consisting diets had been resulted in greater gains in lean body mass compared to subjects on a lactoovovegetarian diet (Campbell et al., 1999).

The animal protein is said to be having a higher contents of protein than the protein in the vegetable plant protein. A high animal protein diets in the daily intake had been shown to give a much greater net protein synthesis than a high vegetable protein diet (Pannemans et al., 1998). This statement supports the information that protein is much better in the protein content rather than the plant protein. Hence the protein content synthesis from the plant is less compare to the content of protein in the animal.

2.1.3 Amino Acid in Animal

Amino acid commonly known to be 20 different types of amino acids those are different from each other. Each different protein is composed of various amino acids put together in varying order with almost limitless combinations. Most proteins are large molecules that may contain several hundred amino acids arranged in branches and

chains (Lauritzen, 1992). The protein sources can be divided into two major classes that are plant sources and animal sources protein. The protein in animal is a complete source of amino acid where all the amino acids that are essential to our body can be taken from the animal sources. This shows that the protein in the plant generally lack one or more amino acids in it. People who want to take benefits from the plant in term of taking the protein from it have to take several type of vegetables or plant to take all the required amino acids in their everyday diet (Hoffman & Falvo, 2004).

For the 20 amino acids that had been identified, they are all needed for human growth and metabolism. However Hoffman and Flavo (2004) stated that in the total of 20 types of amino acids, twelve of these amino acids or eleven in children are nonessential amino acid. The nonessential amino acid meaning that our body can produce the amino acid on our own body without consuming in the everyday diet. The synthesis of the amino acid can be synthesis by the human body by its own without having to be taken from other sources. For the other remaining amino acids, they cannot be synthesized in the body and are described as essential amino acid meaning that they have to be consumed in our diets. Without any of these amino acids, problems will occurs where it will compromise the ability of tissue to grow, be repaired or be maintained (Hoffman & Falvo, 2004).

2.1.4 Types of Amino Acids

There are several types of amino acid that are being the general classification of the amino acids. There are classified based on the different functional group attached to the amino acid with different location with one to another. The carboxylic acid group and amine group attached at the on the same location to the first, or alpha, carbon atom next to the –COOH group (Clark, 2011). The amino acid types can be classified based on the general statement of the requirement on dietary scope of some amino acids. However due to the different amount of the requirement of the amino acid, the classification is hard to be made.

Table 2-1 Twenty Types of Amino Acids with Classification (Volpi et al., 2013)

Essential	Nonessential
Histidine	Alanine
Isoleucine	Arginine
Leucine	Aspartate
Lysine	Asparagine
Methionine	Glutamic acid
Phenylalanine	Glutamine
Threonine	Glycine
Valine	Serine
	Tyrosine

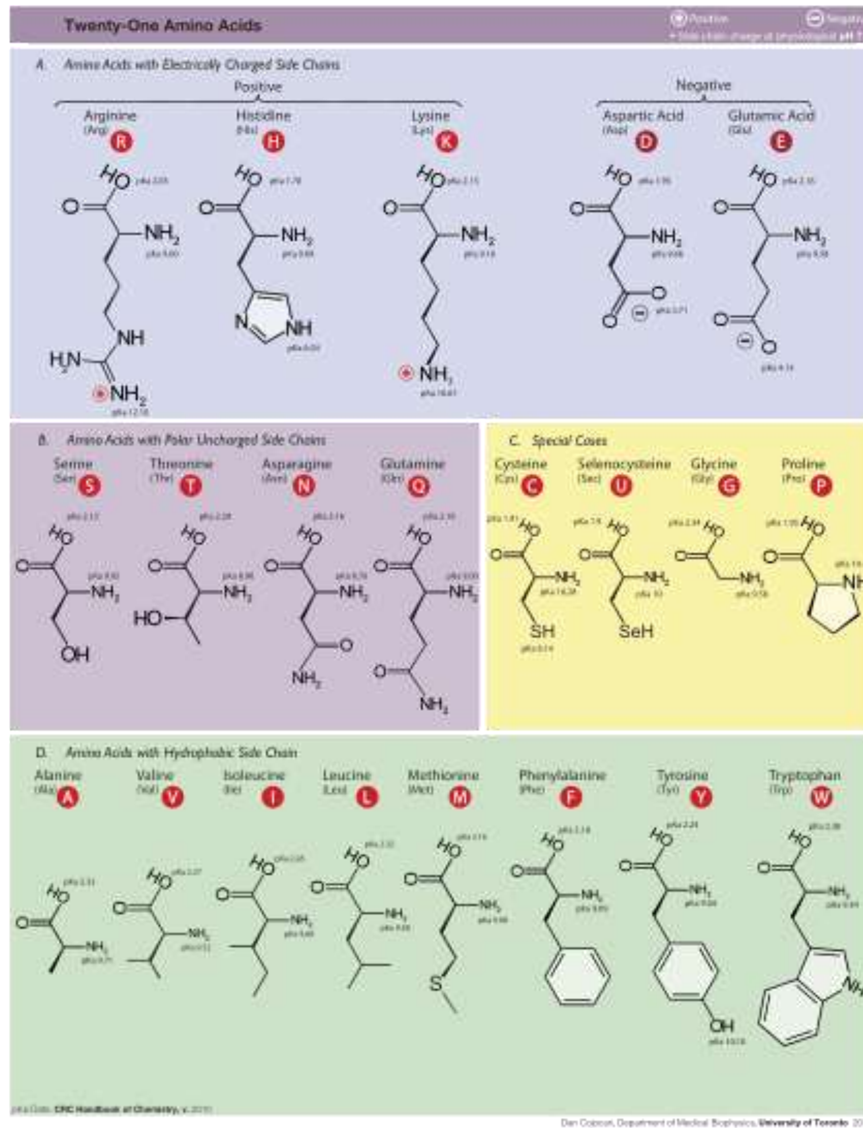

Figure 2-2 Twenty amino acid overview diagram (www.wikipedia.com)

2.2 APPLICATION OF AMINO ACID

The amino acid had been used in many industries especially the food industry. The food industries had been benefited a lot from the technology regarding the amino

acids. The whey protein powder had been using the technology of the amino acid for the usage of throughout food industry (Hoffman & Falvo, 2004). Whey protein is refers to the translucent liquid of milk that remain in the cheese manufacturing. Nowadays the technology of the amino acid had been going step forward in the technology for the athlete muscle building. The applications used based on the amino acid had had been consumed by the athlete as mentioned by Hoffman & Flavo (2004) for the availability of the amino acid that the protein supplies and the way of protein is being utilized.

2.2.3 Advantages of Amino Acid

Amino acid is essential for the building and repair of the muscle of the body. Without these amino acids the process of muscle building will compromised the growing ability of the tissue to be maintained or repaired (Hoffman & Falvo, 2004). The amino acid can be used in many food related industry as an ingredient in the process of used.

2.2.4 Structural View of Amino Acid

Proteins are composed of small units of amino acids. The amino acid that is the building blocks of the protein have limitless combinations where it define the unique type of the type of the amino acid. As been stated by Lauritzen (1992), the structure of the protein is consisted of several hundred of amino acid arranged in branched and chains.

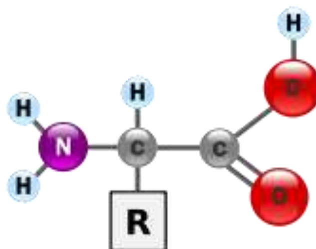

Figure 2-3 Amino acid molecular units

2.3 PROTEIN FROM ANIMAL

Protein can be taken from a lot of sources, especially from the animal sources where it is available everywhere and it is much easier and faster way to obtain the protein rather than plant sources. The sources of protein from animal can be from various part of animal related product. The sources can be taken from eggs, milk, and also from the liquids from animals. *Collocalia fuciphaga* is one of the species of bird that being discover on the quality of protein from the nest produced by these species.

The species of *Collocalia Fuciphaga* had been discussed and research on the availability of the protein and there are many species of swiftlets around the world. The nests of 4 species of swiftlets (*Collocalia* species) have been harvested for human consumption for centuries. These 4 species are *Collocalia fuciphaga, Collocalia germanis, Collocalia maxima, and Collocalia unicolor* (Goh et al., 2001).

2.4.1 Edible Bird's Nest

The edible bird's nest (EBN) is a precious functional food that has been used for several hundred years in China. It is known as the Caviar of the East (Marcone, 2005) in Chinese communities all over the world. EBN mainly comprises a secretion of the salivary gland of several species of *Aerodramus genus* (formerly *Collocallia*) (Jie-Ru, 2009). Due to the highly evaluated function both nutritiously (water-soluble protein, carbohydrate, iron, inorganic salt and fibre) and medically (anti-aging, anti-cancer, immunity-enhancing, etc.), EBN has been esteemed a precious food tonic by Chinese people ever since the Tang dynasty (618 AD) (Yajun, 2010).

Figure 2-4 *Collocalia fuciphaga* nest

Usually "Edible bird's nest" refers to the nest made by several different swiftlet species. Human consumption of these nests has been a symbol of wealth, power, and prestige, as well as being used medicinally in traditional Chinese medicine (Marcone, 2005). The nest is essentially woven by Swiftlet or *Collocalia* from strands of gelatin of its saliva mixed with minor feathers. Traditionally, EBN has been considered nourishing and a promoter of health for the sick and aging (Qin, 2000). Guo (2006) stated that

Chinese have been consuming bird nests for hundreds of years, and most of them believe that EBN consists of several proteins and minerals that promote the generation and growth of human cells, rejuvenate human skin, and strengthen the immune system.

According to Goh et al. (2001), EBN originates from the saliva of one of four swiflets (*Collocalia* species). These 4 species are *Collocalia fuciphaga*, *Collocalia germanis*, *Collocalia maxima*, and *Collocalia unicolor* (Lau & Melville, 1994). These swiflets are found only in the Southeast Asian region. The nests built by male swiftlet during breeding season are made almost entirely from saliva produced by their sublingual salivary glands. Some species include feathers in their nests, but these amounts are at most to 10% of the dry weight. The nests are composed mainly of glycoprotein. The carbohydrate component consists of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose (Goh et al., 2001). The most abundant amino acids present are serine, threonine, aspartic acid, glutamic acid, proline, and valine. One study has shown the presence of a glycoprotein capable of promoting cell division, and another has demonstrated the presence of an epidermal growth factor–like protein (Kong et al., 1987)

2.3.1.1 Nutritional Value of EBN

Analysis had been made nowadays to determine the nutrition on the Edible Bird Nest. On the Table 2.2 below, comparison had been made based of the analysis of several type of processed EBN sample from 4 commercial brands of processed EBN. From the analysis that had been made the crude protein was the most abundant

composition than the other composition of material that is found on the analysis. Norhayati et al. (2010) stated that EBN is a good source of protein and minerals and can have the benefits in the nutritional aspects. Thus the data agree with the statement where the composition of protein detected is high in EBN. The amino acid composition of the edible nest can be seen in the Appendix C.

Table 2-2 Mean (SEM) concentration of protein, minerals and sialic acid in processed EBN samples (Norhayati et al., 2010).

	Brand X (n=2)	Brand Y (n=2)	Brand A (n=2)	Brand B (n=2)	Unprocessed EBN (n=18)
Crude Protein (g/100g)	58.7(2.8)	56.2(0.7)	61.5(4.0)	56.7(0.2)	61.5(0.6)
Minerals (mg/ 100g)					
Calcium	2071.3(37.4	2071.3(16.3	503.6(4.9)	524.8(5.7)	553.1(19.5)
Sodium	110.8(2.2)	39.8(0.9)	509.6	505.9(0.2)	187.9(10.4)
Magnesium	79.0(0.4)	67.5(2.1)	97.0(1.0)	99.6(1.2)	92.9(2.0)
Potassium	7.0(0.7)	33.7(3.7)	107.2(0.1)	75.2(8.8)	6.3(0.4)
Phosphorus	4.1(0.1)	4.2(0.5)	12.5(1.9)	7.7(0.5)	2.3(0.5)
Iron	2.9(0.3)	2.2(0.7)	0.9(0.2)	2.0(0.7)	1.2(0.1)
Zinc	1.3(0.0)	1.0(0.0)	1.4(0.6)	0.7(0.1)	0.9(0.1)
Copper	0.4(0.0)	0.4(0.0)	0.4(0.0)	0.3(0.0)	0.5(0.0)
Glycoprotein (%) Sialic Acid	ND	1.5(0.9)	0.7(0.2)	0.7(0.2)	0.7(0.1)
ND - not detectable					

2.4.2 Reason Selection of Animal Protein

The reason for the selection of animal protein is because of the composition of the protein available in the sources is higher compare to the other sources. Below is the table for the comparison of the protein content of in selected type of food.

Table 2-3 Protein quality ranking (Hoffman & Falvo, 2004).

Protein Type	Protein Efficiency Ratio	Biological Value	Net Protein Utilization	Protein Digestibility Corrected Amino Acid Score
Beef	2.9	80	73	0.92
Black Beans	0		0	0.75
Casein	2.5	77	76	1.00
Egg	3.9	100	94	1.00
Milk	2.5	91	82	1.00
Peanuts	1.8			0.52
Soy protein	2.2	74	61	1.00
Wheat gluten	0.8	64	67	0.25
Whey protein	3.2	104	92	1.00

2.4.3 Problem Related on Protein Consumption

The protein from animal is usually containing high concentration of protein. However some consideration on the protein content whether it is suitable for the utilization of all sort of people. There are some problems that come out from the consumption of protein in some cases. Due to the some religious matter that comes out, Ng & Mohd Khan (2012) had stated that this matter had grown up in the issues of the cholesterol contents of the animal protein and alternative for the plant protein as the other sources of the protein (Ng & Mohd Khan, 2012). However Hoffman & Falvo (2004) stated the protein in plant had been considered as low content of protein inside rather than protein from the animal sources.

2.4 PROCESS PROTEIN PRODUCTION

The production of protein might be from several method of extraction. There are physical, chemical and biological methods of protein extraction. The productions of protein are affected from various parameters. The protein solubility can be affected from the parameters such as pH, temperature, ionic force, salt or solvent type, extraction time, solid-solvent ratio, presence of components causing linking (Mitzubuti, 2000). This solubility of a protein, and its functionality as a nutritional ingredient, may be influence by extraction conditions, solvent type and heat treatment.

The protein production may be done in several methods. There are methods by physical, chemical and biological methods that had been used in the process of the treatment to get the protein in the sample that are being treated. The list for the available treatment that can be applied can be seen in Table 2-4.

Table 2-4 Pre-treatment methods for enzymatic hydrolysis (Taherzadeh and Karimi, 2007).

Processes	Processes	Mechanism of changes on biomass
Physical pre-treatment	- Ball-milling - Two-roll milling - Hammer milling - Colloid milling - Vibro energy milling - Hydrothermal - High pressure steaming - Extrusion - Expansion - Pyrolysis - Gamma-ray irradiation - Electron-beam irradiation - Microwave irradiation	- Increase in accessible surface area and size of pores - Decrease of the cellulose crystallinity and its degrees of polymerization - Partial hydrolysis of hemicelluloses - Partial depolymerization of lignin
Physicochemical & chemical pre-treatment	Explosion: - Steam explosion - Ammonia fiber explosion (AFEX) - CO2 explosion - SO2 explosion Alkali: - Sodium hydroxide - Ammonia - Ammonium Sulfite Gas: Chlorine dioxide - Nitrogen dioxide Acid: - Sulfuric acid - Hydrochloric acid - Phosphoric acid - Sulfur dioxide Oxidizing agents: - Hydrogen peroxide - Wet oxidation - Ozone Cellulose solvents: - Cadoxen - CMCS	- Delignification - Decrease of the cellulose crystallinity and its degrees of polymerization - Partial or complete hydrolysis of hemicelluloses
Biological pre-treatment	- Actinomycetes - Fungi - Enzyme	- Delignification - Reduction in degree of polymerization of hemicellulose and cellulose

2.4.1 Biological Method : Enzymatic Hydrolysis

According to Martina et al. (2001), compared to acid or alkali hydrolysis on enzymatic hydrolysis of protein, using selective proteases provides more moderate conditions of the process and few or no undesirable side reactions or products. In addition, the final hydrolysate after neutralization contains less salts and the functionality of the final product can be controlled by selection of specific enzymes and reaction factors (Darwicz et al., 2000). Nowadays, Enzymatic adjustment of proteins using particular proteases to break down specific peptide bonds had been widely been used. The peptides produced have a smaller molecular size than proteins. Thus, their functional properties are different: increased solubility over pH range, decreased viscosity, and significant changes in foaming, gelling and emulsifying properties.

2.5 PARAMETERS

2.5.1 Effect of pH on the protein concentration in Enzymatic Hydrolysis.

Based on research of Jiang et al (2010), pH is being a significant process parameter which pH influences a lot to both enzyme activity and protein solubility. Mune et al. (2008) also found that increase of pH and NaCl concentration leads to an increase of protein yield. In one research conducted by See et al. (2011), the protein yield increased with an increase in substrate pH up to pH 8.39, beyond which, the yield of protein decreased. This statement was agreed with the working pH range of Alcalase since it is an alkaline enzyme with optimal pH of 8 – 8.5.

2.5.2 Effect of Temperature on the protein concentration in Enzymatic Hydrolysis.

Hydrolysis temperature has a big influence on free oil yield and protein hydrolysates yield. The best temperature of Alcalase ranges from 55^oC to 65^oC and will be increased as the temperature increased. However, once the temperature is beyond the optimum temperature, the trend will be decreasing as stated by See et al., (2011) where the degree of hydrolysis of protein was raised with an increasing temperature but exhibited a decreasing trend from the temperature of 55.3°C to 70°C.

2.5.3 Effect of Enzyme loading on the protein concentration in Enzymatic Hydrolysis.

The product of the protein hydrolysates yield increased along with the increase in the amount of enzyme. More so, the extent of the increase is larger between 0.5% and 1.0%. The more enzymes are added, the more sufficient the reaction of the enzyme and the protein substrate, and the more the rupture of peptide bonds (Jiang et al, 2010). In one research conducted by See et al. (2011), the rate of hydrolysis of protein was increased as the enzyme was added. The increase of enzyme to substrate level showed that it will cause the degree of hydrolysis (DH) of the protein will be increased.

2.6 ANALYSIS METHODS

2.6.1 DNS method

DNS method is a new method that being used to estimate the amount of reducing sugar in sample. In the recent days, most of the new methods used to determine cellulase activity via the DNS principle was that after the enzymatic hydrolysis of the cellulose was formed. In this method, the reducing sugar can reduce the nitro of 3,5-dinitrosalicylic acid (DNS) to amino acid , in this manner generating a reddish brown colour for amino compounds (Shuangqi et al., 2011) The relationship of the amount of reducing sugar and the brown colour of the solution shows the positive correlation between them. The cellulose can be estimated by the spectrophotometric method by UV-Vis Spectrophotometer device.

2.6.2 Lowry Method Analysis

The method that was proposed by Lowry et al. (1951) has been most widely being the method to estimate the protein amount in biological sample. The assay is already in solution or can be in easily-soluble dilute alkali. First the proteins are pre-treated with copper ion in alkali solution, and then the aromatic amino acids in the treated sample reduce the phosphomolybdate phosphotungstic acid present in the Folin Reagent. The end product of this reaction has a blue colour. The amount of proteins in the sample can be estimated via reading the absorbance (at 750 nm) of the end product of the Folin reaction against a standard curve of a selected standard protein using bovine serum albumin. The method of analysis using the Lowry Method can had been mostly used nowadays.

2.6.3 Fourier Transform Infrared Spectroscopy

The Fourier-transform infrared spectrometer gives an absorbance spectrum that can detect much higher absorbance than the UV-Visible spectrophotometer. The FTIR acquires an absorbance spectrum without using a monochromator. Infrared spectrometers used to have monochromators, but the spectra were usually noisy because infrared detectors in are relatively insensitive. This is because thermal background noise is large in the infrared. The method of Fourier-transform was introduced to ease the disadvantage of the background noise.

Infrared spectroscopy has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum.

Infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis.

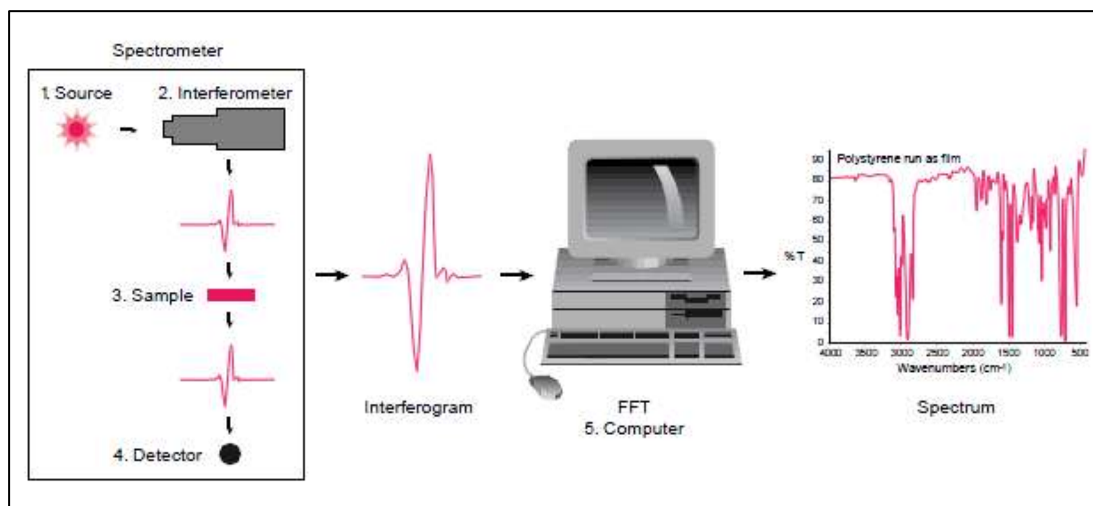

Figure 2-5 FTIR Sample analysis process

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The process of the determination of protein in this research consist several stages. In this particular research, the process of the determination of the protein concentration can be categorized into three main stages. The stages that had been undergone are as the overall view of how is the experiment will be going to be done. The first stage of the experiment is the pre-treatment stage. The raw material was at first undergo treatment where the impurities in the sample had been removed so that the sample will be as the original as it is produced. The raw material that had been taken will be undergoing pre-treatment before it will be hydrolyze in the second stage. The second stage is the enzymatic hydrolysis stage where the process will be on the extraction of the sample with some addition of enzyme in the sample solution (See et al., 2011). Then the process will be the separation of the sample. The sample will be separate so that the liquid and the solid particles will be separated from each other to go

to the next stage for the determination of protein. The next stage is the process of the analysis of the protein content on the sample. All of these stages should be done in the determination of the protein in the sample in this particular research. For the overview process of the research, the outline for the experiment can be concluded as below;

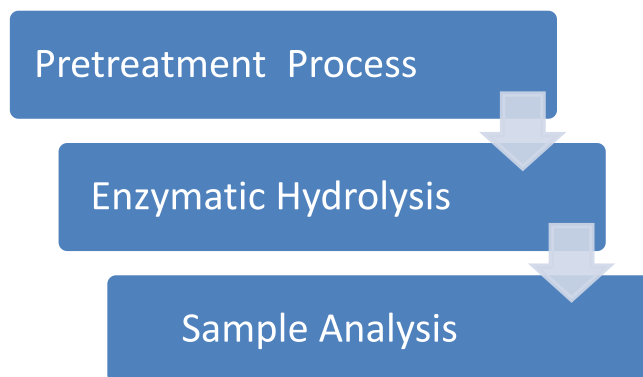

Figure 3-1 Overall stages of research

3.1.1 Stage I : Pre-Treatment Stage

In this stage the sample of the experiment that is the Edible Bird Nest (EBN) is being treated to get the pure sample. During this pre-treatment stage, the process will be done to take only the pure nest without any impurities. Firstly, the nests will be cleaned by soaking them in water. The process had been done until the nest cement is softened and the tightly bound laminae partially loosen (Norhayati, 2010). Then the sample will be filtered out and the filtrate will be removed. The small feathers and line plumage then will be manually removed with tweezers and toothpicks.

Figure 3-2 Untreated raw material (*Collocalia fuciphaga* bird nest)

The purpose is to make the nests as if it is first constructed by the swiftlet without impurity. The cleaned bird's nests are freeze-dried/dried using a fan. Throughout the process, no additives, bleaching agent and chemicals are adulterated into the bird nests (Massimo, 2005).

Figure 3-3 Pre-treatment of raw material

Figure 3-4 Removal of feathers and plumage

Figure 3-5 Sample filtrations

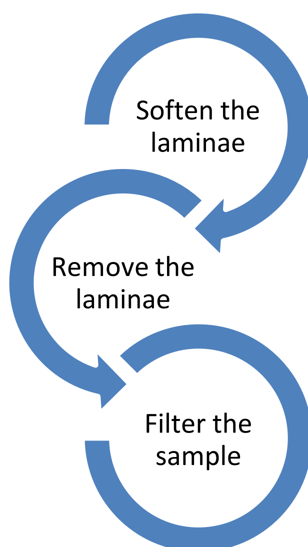

Figure 3-6 Overview process of sample pre-treatment

3.1.2 Stage II : Enzymatic Hydrolysis Stage

The next of the research is the Enzymatic Hydrolysis stage. This stage is the most crucial part important part of the experiment where all the extraction and separation process is being done in this stage. This stage will proceed after the sample had been undergoing the pre-treatment process when all the impurities had been removed. This stage will be on the following steps of processes. The first step will be on

the extraction step which the sample will be undergoing the hydrolysis where the cell wall will be broke down and the protein will be recover in this step. Enzymatic hydrolysis had been proven to be the most efficient method to be done in order to extract protein and to recover the protein and have the higher potential of commercial values (Nguyen et al., 2011).

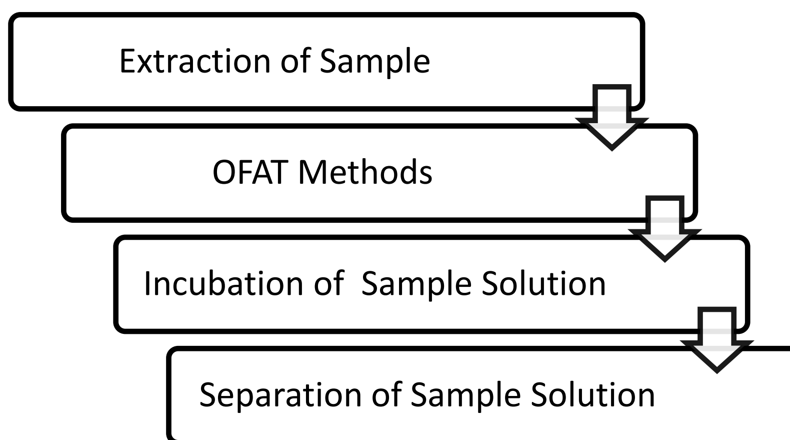

Figure 3-7 Overview process of enzymatic hydrolysis

3.1.2.1 Extraction Process

The extraction is done by the processes to degrade the protein to a smaller fraction so that it can be absorb easily to the body. In this process the enzyme is added to assists and to increase the rate of hydrolysis of the sample. The extraction process can be described in the diagram below.

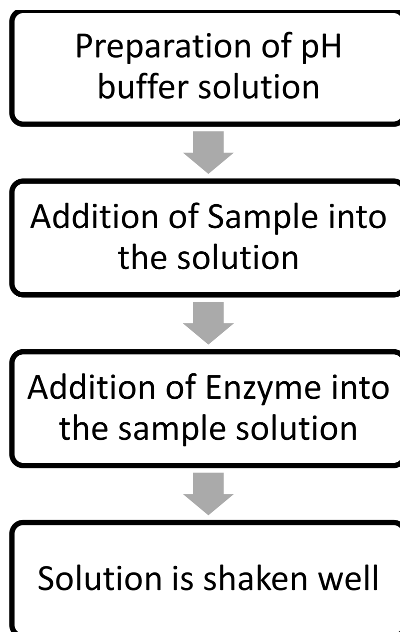

Figure 3-8 Extraction process of sample

3.1.2.2 Enzyme Addition

The sample has been added with enzyme to increase the hydrolysis rate of the sample. Protease enzyme is one of the enzymes that have the good ability to solubilize protein. The time for the enzyme activity was being limit so that the enzyme will not react to the entire process during the extraction. In this research the enzyme that mainly will be used is protease. Protease used in this experiment is Alcalase 2.4L.

According to Chabeaud, et al. (2009), Alcalase has great ability to solubilize protein with an optimum temperature that ranged from 50 to 70°C and has optimal pH range at the value of 8 to 10. The optimum activity for Alcalase has also been found to be under the following conditions: pH between 8.0–9.0 and temperature between 50–60°C as assured by Rosenthal et al. (2001). Also in some other statement shows that by

the comparison to acid or alkali hydrolysis, enzymatic hydrolysis of protein, by using selected protease, gives more moderate environment of the process by providing with few or even without undesirable products and side reactions (Hrčková & Zemanovič, 2002).

3.1.2.3 One Factor at a Time (OFAT) Method

The process of the enzymatic hydrolysis has many factors affecting the rate of protein produced by the extraction. In several other researches, the parameters that had been used are the extraction time, temperature, solution concentration and also enzyme loading (Wani et al., 2008). In this research the parameters that had been done is by using two different parameters that are pH and the enzyme loading. The temperature parameter had not been done because of the enzyme used and can be stand up to a high temperature up to 60°C. The only two parameters that had been chosen had been undergoing the method of One Factor at a Time (OFAT) in order to check the factor that affect the yield of protein production and condition that has the highest protein production.

Figure 3-9 Extraction process

3.1.2.4 Incubation of Sample Solution

The sample was incubated in the incubator shaker in 50°C for 1 hours of incubation time (Rosenthal et al., 2010). The speed was set up to 150 rpm and the sample was closed with aluminum foil for safety precaution. The enzyme is then inactivated by keeping the mixture in the water bath at 93-94°C for three minutes (Palupi et al., 2010). The slurry is then cooled down to a room temperature before went for the next process of separation the mixture.

Figure 3-10 Water bath BS-21

3.1.2.5 The Separation of the Mixture

The mixture then was separated in the centrifuge to separate the mixture into 2 different phase of liquid and solid phase. The supernatant liquid will be taken out and the solid sediment is removed and kept for further analysis (Shen et al., 2008)

3.1.3 Step III : Sample Analysis

Then the last stage is the sample analysis stage where the sample is being analyze for the protein concentration. In this stage the supernatant solution that had been undergoing the separation process will be going to be analyzed using the UV-Vis spectrophotometer to determine the protein content on the sample that had been treated and going the extraction process. All of the supernatant liquid from the sample that that had been going the separation process will be analyzed for protein concentration using UV-Vis spectrophotometer. The higher density of the sample exhibits the higher protein yield from the extraction process.

3.2 MATERIALS

3.2.1 *Collocalia fuciphaga*

The raw material that will be chosen in this research is the edible bird nest from *Collocalia Fuciphaga* species that was taken from Pekan, Pahang. The nests will be cleaned by soaking in water to soften nest cement. The nest will be filtered using filter paper to remove excess water. Feathers and fine plumages from the sample will be manually removed with forceps. The nests were allowed to dry and the EBN samples were transferred into Scott bottle and kept at ambient temperature until further usage (Norhayati, 2010).

3.2.2 Protease Enzyme

The protease enzyme that had been used was Alcalase 2.4 L. The enzyme used is a microbial protease taken from *Bacillus Licheniformis* with endopeptidase activity. The enzyme was provided by Novo Nordisk (Bagsvaerd, Denmark) with a main component is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 AU (Anson Unit) per gram.

3.3 METHODS

3.3.1 Prepare the Standard Protein Curve

A different dilution of Bovine Serum Albumin (BSA) solutions will be prepared (Table 3.1) by mixing the BSA with water in the test tube. The final volume in each of the test tubes is 5 ml. The BSA range is 0 to 1.0 g/L. The dilution will be prepared by the following recipe at the table shown below:

Table 3-1 Dilution from the BSA solution (1.0 g/L) for the standard curve

Volume distilled water, L	Mass of BSA, g	Final concentration, g/L
0.01	0	0
0.01	0.002	0.2
0.01	0.004	0.4
0.01	0.006	0.6
0.01	0.008	0.8
0.01	0.01	1.0

3.3.2 Preparation of Buffer Solutions

In order for the enzyme to react at the optimum condition, the pH buffer had been prepared with several pH values from pH 7 to pH 10. The preparation for the buffer had been made using several chemical that had been prepared using specific proportion. The preparation of the Potassium Phosphate Buffer and Glycine-NaOH Buffer by mixing of solution from different chemical can be seen at the Appendix 1.

3.3.3 Protein Extraction

There are two parameters that has been control in this research. The parameters are the pH of the extraction solution and the enzyme loading. In this research, the one-factor-at-a-time method (or OFAT) will be applied for all of experiments. The protease enzyme loading that will be used is ranged from 1.5% (v/w) to 3.0 (v/w) for all the solution of material over 10 ml of buffer and 1 gram of sample. The extraction temperature was fixed at 50°C for all of the parameters. The pH used was varies ranging from pH 7 – to pH 10 (Table 3.2) using the pH buffer that had been prepared. The extraction is occurring inside the test tube and the tube is shaken in water bath for 1 hour (Rosenthal et al., 2001).

Before protein assay were made, the Modified Lowry and Folin-Ciocalteu reagents will be prepared. The Modified Lowry reagent will be prepared by adding the Reagent A (20 g Na_2CO_3 + 4 g NaOH dissolved in 1 liter distilled water) and Reagent B (2.5 g $CuSO_4.5H_2O$ + 5 g sodium citrate dissolved in 1 liter distilled water) in the proportion of 50:1. While the Folin-Ciocalteu reagent will be prepared by diluting the supplied 2X reagent (2N) 1:1 with distilled water (Lowry et al., 1951).

From the different buffer solutions that had been made, 1 gram sample had been added into different test tubes. Then 1.0 ml of the Modified Lowry reagent will be added into the test tubes. After the solution is mix well, the solutions will be incubated at room temperature for 10 minutes. At the end of the incubation period, 0.1 mL of 1 N Folin-Ciocalteu reagent, which will be bought from Merck Darmstadt Germany will be added into the reaction mixture and left at room temperature for 30 minutes.

Table 3-2 Data for the one factor at a time (OFAT)

pH	Enzyme/ Loading	Temperature (^{o}C)
6	1.0	50
7	1.5	50
6	2.0	50
9	2.5	50
10	3.0	50

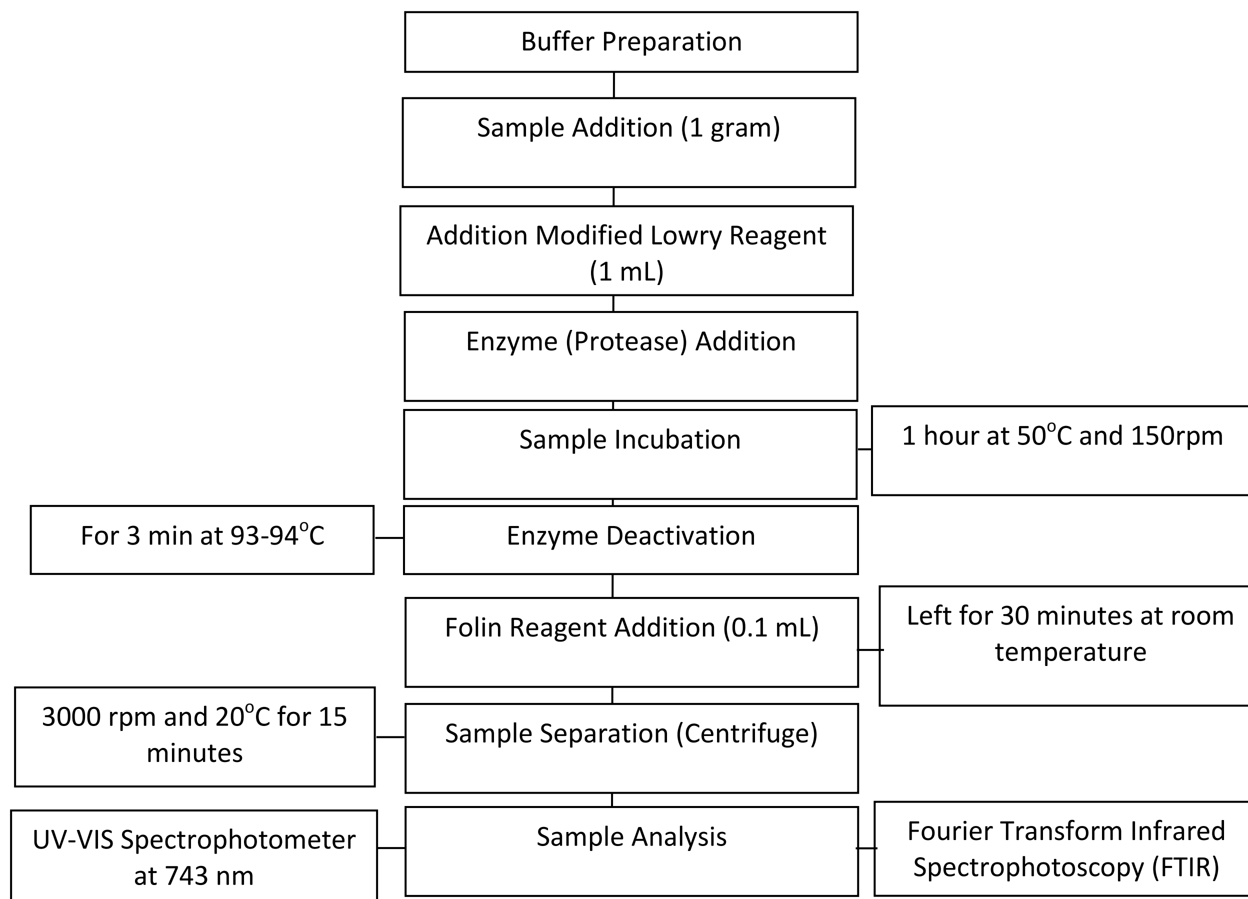

Figure 3-11 Flow of the research methodology

3.3.4 Solid-Liquid Separation

Following extraction, the suspension will be centrifuged under 3000 rpm for 15 min at 20°C in a Sorvall RC5C centrifuge. The precipitate will be removed, and the supernatant will be taken for moisture content determination (Rosenthal, *et al*., 2001).

Figure 3-12 Sorvall RC5C centrifuge

3.3.5 Determination Protein Concentration in Sample

After 30 minutes, the sample will be analyzed using the UV-vis spectrophotometer model Hitachi U-1800 in order to determine the concentration of protein in each of the sample. Distilled water will be used as a blank. The colorimeter had been set to zero with blank and the optical density had been taken by measuring the absorbance at 743 nm. The absorbance of the sample will be measured at 743 nm. A standard curve will be prepared by plotting the average blank corrected 743 nm reading for each BSA (bovine serum albumin) standard, which had been purchased from Merck Darmstadt Germany versus its concentration in g/L. The protein concentration of the unknown sample will be determined for each sample using the standard curve. (Palupi et al., 2010).

The amount of soluble protein in the filtrate will be determined by the method of Lowry et al. with bovine serum albumin as standard (Palupi et al., 2010). The supernatant were pipette out to semi micro disposable cuvettes and the concentration of

the protein is measure by using the UV-Vis spectrophotometer. The graph standard protein versus temperature will be plotted. Then, the new range of temperature, pH and enzyme loading for *C.fuciphaga* extraction is taken from the graph.

Figure 3-13 UV-Vis Spectrophotometers

3.3.6 Characterization of the Sample Using Fourier Transform Infrared Spectroscopy (FTIR)

The sample had been analyzed in order to characterize the element in sample to meet the required standard of the study. The supernatant liquid of the sample that had been kept had been brought to analyze using the FTIR device. The results from the sample analyzed had been compared with the standard protein solution of Bovine Serum Albumin. The graph obtained from the analyzed sample using the device will be compare with the table of the element wavelength.

Figure 3-14 Sample characterization using FTIR

FTIR (Figure 3.14) was used to separate the individual frequencies of energy emitted from the infrared source. This was accomplished by the use of a prism or grating. An infrared prism works exactly the same as a visible prism which separates visible light into its colors (frequencies). A grating is a more modern dispersive element which better separates the frequencies of infrared energy. The detector measures the amount of energy at each frequency which has passed through the sample. This results in a spectrum which is a plot of intensity vs. frequency (Mariey et al., 2001).

3.3.7 Flow Diagram on Overall Process

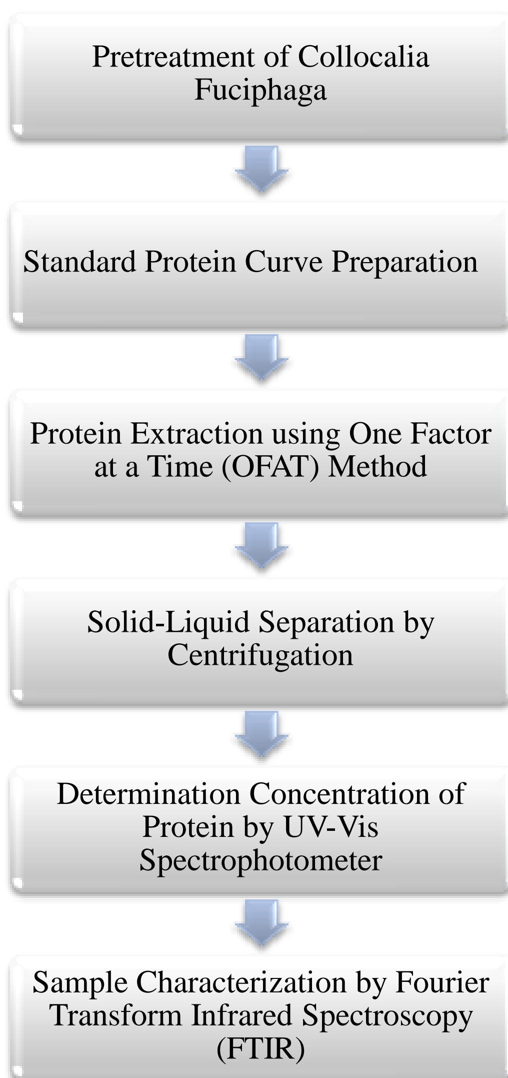

Figure 3-15 Overall process on research methodology

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In order to obtain and determine the protein concentration in the samples that need to be analysed, the standard curve of the protein standard has been made. The purpose of the standard curve that has been made is to be a reference for the concentration of the protein in the sample. Once the graph of the standard curve had been plotted, the protein concentration in the sample can be determined by comparing to the standard curve that had been prepared earlier. The analysis that had been made was analysed mostly by using Lowry's assay method (Lowry et al., 1951). The other analysed method in determination of overall protein concentration also could be done such as Kjeldahl method. By using the Lowry's assay method, the absorbance was read at 743 nm using the UV-Vis Spectrophotometer device where the standard curve were prepared before and the results were calibrated against protein standard solution up to 1 g/L.

4.2 PROTEIN STANDARD CURVE

The standard had been made by using the dilution of the BSA (Bovine Serum Albumin). The dilution that had been made was from 0 mg/L, 0.2 mg/L, 0.4 mg/L, 0.6 mg/L, 0.8 mg/L and 1.0 mg/L of BSA solution. The graph had been made by plotting the optical density (OD) of the sample versus the concentration of the BSA. The straight line had been drawn on the best fit to make the standard for the protein concentration determination as a reference.

Table 4-1 Optical Density of Different Concentration of Bovine Serum Albumin

Concentration of BSA Solution g/L	0	0.2	0.4	0.6	0.8	1
Optical Density (OD)	0	0.422	0.566	0.696	0.991	1.125

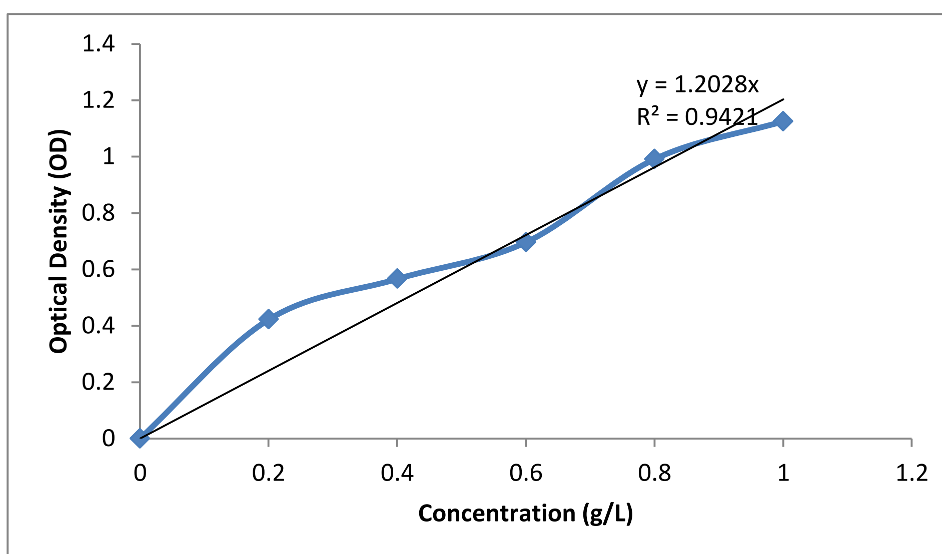

Figure 4-1 Optical Density (OD) versus BSA concentration

4.3 EFFECTS OF PH ON THE PROTEIN CONTENT

The first parameter that had been done is the pH manipulation of the buffer solution of the sample solution. The results that had been taken had been taken and had been tabulated (Table 4-2).

For the experiment results, the expected outcomes should get the data close to the results as discussed on the journals related on the topic. The maximum protein concentration is obtained at the pH 8 to 9. Based on the previous research, the yield of protein for the experiment on manipulating the pH of the mixture should be increase as the pH is increased as stated by See et al. (2011) and the hydrolysis rate was raised with the increasing of substrate pH up to pH 8.39. The optimal pH range of Alcalase is said to be at value of pH 8 to pH 10 and thus could reduce the risk of microbial contaminations (See et al., 2011). This statement was agreed by Shen et al., (2008) that Alcalase had the highest extraction rate at pH 8.0. Also in the other research according to Wani et al. (2006), the current study agreed that the increase in pH resulted in higher protein concentration yield.

Table 4-2 summarizes the protein concentration yield when hydrolysis of different buffering capacities was tested. The results are presented for peptide bond cleavage at constant temperature of 50°C and pH increase from pH 7 to pH 10 in experiments performed under same time of extraction and enzyme loading of 25 micro litre.

Table 4-2 Concentration of protein from samples from extraction on different pH solutions

pH	Trial	7	8	9	10
Concentration (g/L)	1	0.2072	0.3794	0.3993	0.2995
	2	0.1747	0.4143	0.3968	0.3794
	3	0.1423	0.3943	0.4493	0.3394

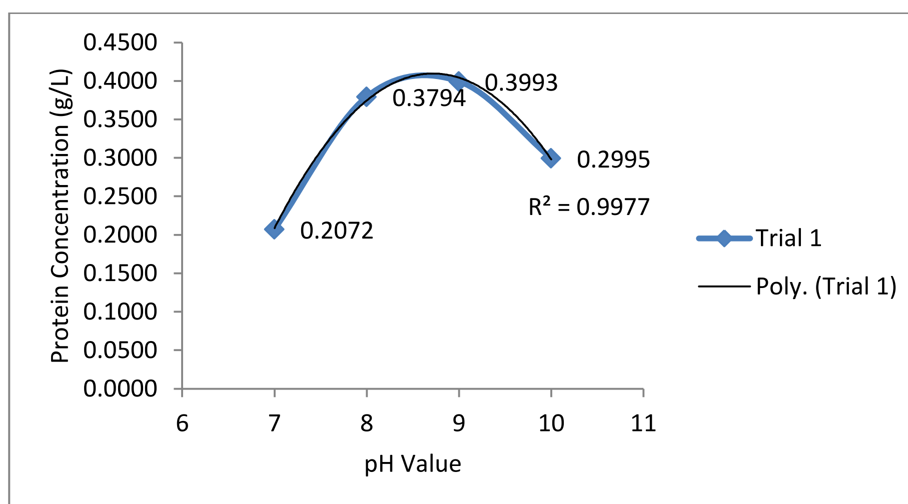

Figure 4-214 Protein concentration versus pH of the sample solution in first trial

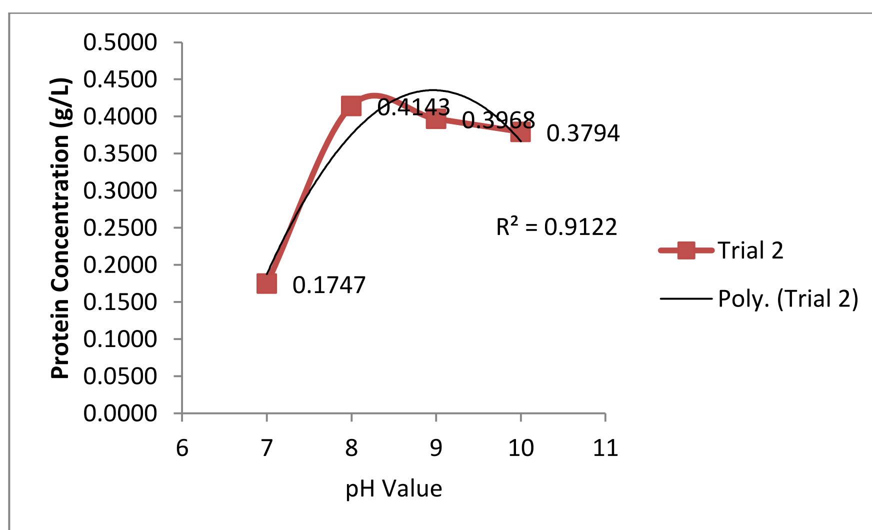

Figure 4-3 Protein concentration versus pH of the sample solution in second trial

The protein concentration is increasing when the pH value reach between pH 8 and pH 9 in both of trial 1 and 2. The trend can be seen in the Figure 4-2 and Figure 4-3 which the highest protein concentration is analysed at pH 8 and pH 9 respectively. Hence this data prove the previous research that had been done before. The progress of the curve is decreasing when the pH of buffer solution is increase because of the factor that the optimum condition for the enzymatic hydrolysis of protein using Alcalase lays between pH8-8.5 (See et al, 2011).

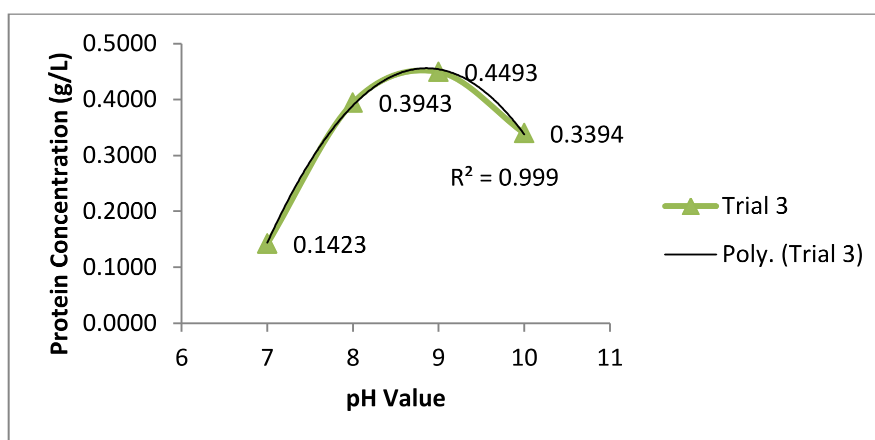

Figure 4-4 Protein concentration of versus pH of the sample solution in third trial.

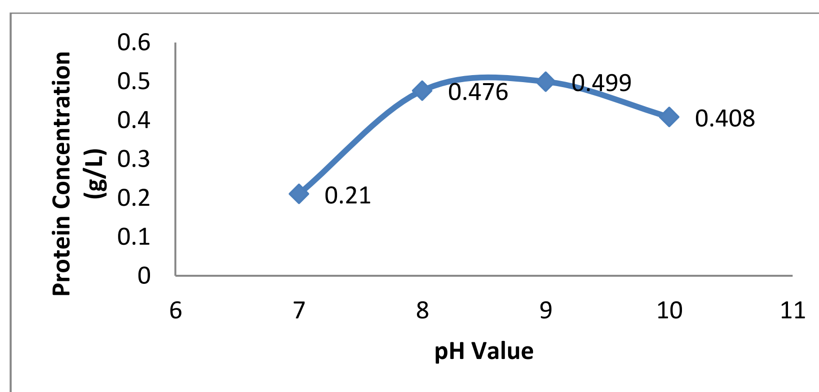

Figure 4-5 Average value on protein concentration yield on different pH

This result obtained was supported by FTIR analysis which shows that the optimum pH exhibited similar pattern with by using the UV-VIS analysis with reference to the BSA standard. The protein compound had been detected on the sample by using FTIR devices. The difference whereas can be detected by analysing different solution of sample and the absorbance of the compound detected is increasing throughout the increasing of the pH of the solution. The absorbance detected for the lowest pH of the solution shows that the extraction process was at the lowest degree of hydrolysis because of the optimum extraction process for the hydrolysis using Protease enzyme will be at the pH 8 to pH 10 as being agreed by several researches (See et al, 2011, Shen et al, 2008).

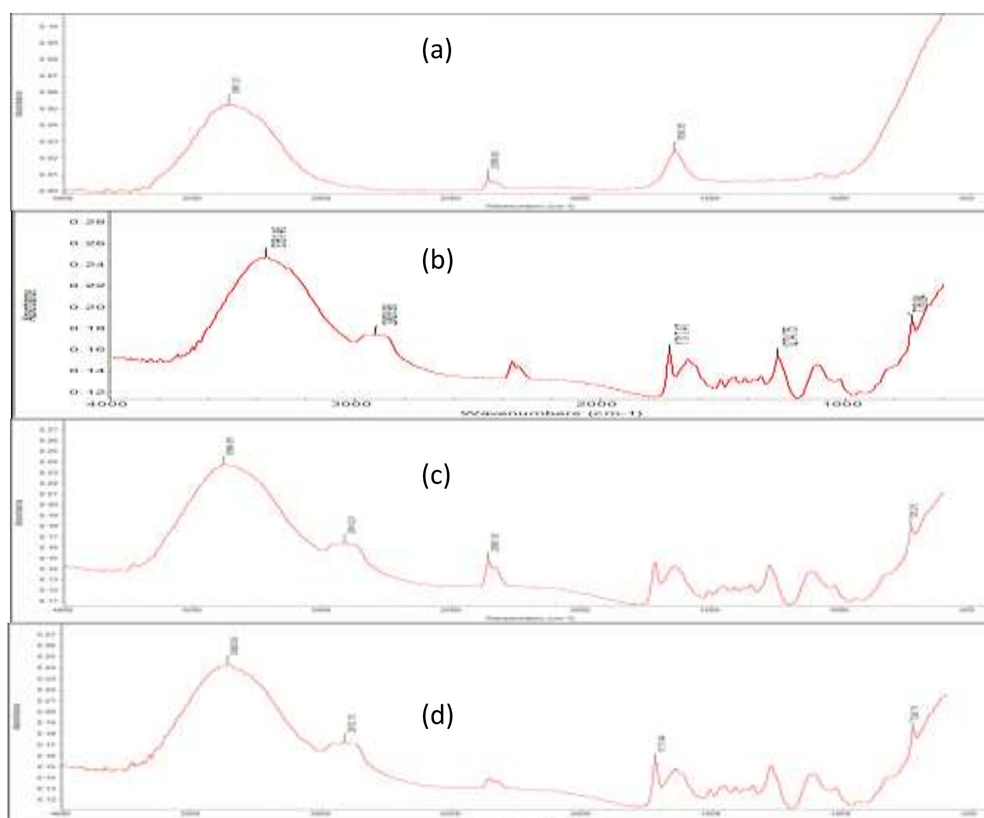

Figure 4-6 FTIR analysis results on different pH solution. (a) pH7 (b) pH8 (c) pH 9 (d) pH 10

In addition to the statement, Kain et al. (2009) had been stated that the protein solubility was dependent with the pH changes, where in this study the pH had affected to gradual increasing in protein concentration from pH 7 to pH 8. Thus based on 3 replicates of sample studied the optimum pH is found to be at pH 8.5. As pH is increases, the particles become closer due the net negative of columbic forces between negative and positive charges of the protein residues (Boulet et al., 2000). However the van der Waals attraction and also the hydrogen bonding hold the mass together against the forces to make the solubility of the protein is to be increase (Ng et al., 2012).

4.4 EFFECTS OF TEMPERATURE TO THE HYDROLYSIS PROCESS

The yield of protein for the experiment on manipulating of temperature should be increasing as the temperature is increasing. Palupi et al. (2010) had agreed by the statement where the protein concentration was raised with the higher temperature increased up to the optimum temperature. However, the protein concentration exhibit a decreasing trend from 55.3^{o}C to 70°C because the optimal temperature of Alcalase enzyme is 55°C and could achieved the highest protein yield at temperature 60°C .A temperature which is too high or too low would either kill or inactivate enzymes (Shen et al., 2008). Alcalase has great ability to solubilize fish protein and is nonspecific, with an optimum temperature that ranged from 50 to 70°C. Hence in this research the temperature is not discussed as the high range of the optimal temperature of the enzyme could be.

4.3 EFFECT OF ENZYME LOADING ON THE PROTEIN YIELD

The protein yield for the manipulation of the enzyme concentration shows that the yield for the protein is that either too low or too high enzyme concentration is not desirable for protein extraction (Shen et al., 2008). Basically, there should be an optimum concentration of enzyme to produce maximum yield of protein. Shen et al., (2008) conclude that the extraction yield will increase undoubtedly as the enzyme concentration that will normally enhance the protein solubility on the solvent. However, the existence of inhibitor from the extracted protein molecules combining with the enzyme prevents the activity of the hydrolysis.

Table 4-3 Concentration of protein yield from sample from extraction with different enzyme loading

E/L (%)	Trial	1.5	2	2.5	3
Concentration (g/L)	1	0.3727	0.3478	0.3428	0.3511
	2	0.3627	0.3361	0.3644	0.3594
	3	0.3527	0.3594	0.3527	0.3552

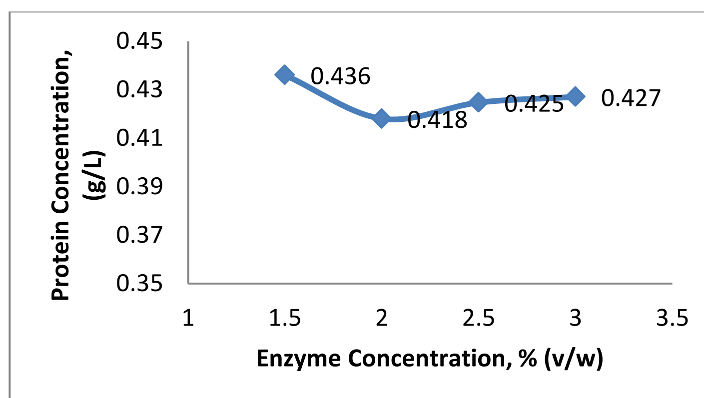

Figure 4-7 Concentration of protein versus enzyme loading (%) of the sample solutions in first trial.

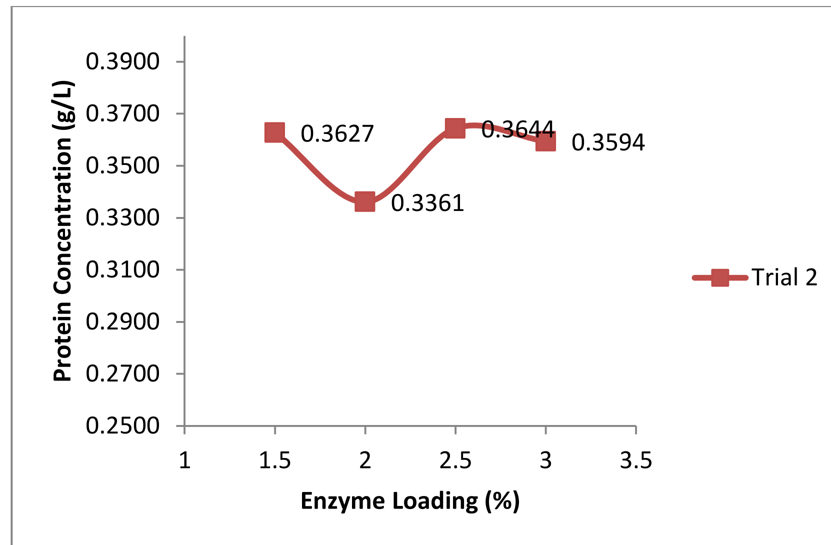

Figure 4-8 Protein concentration versus enzyme loading (%) of the sample solutions in second trial.

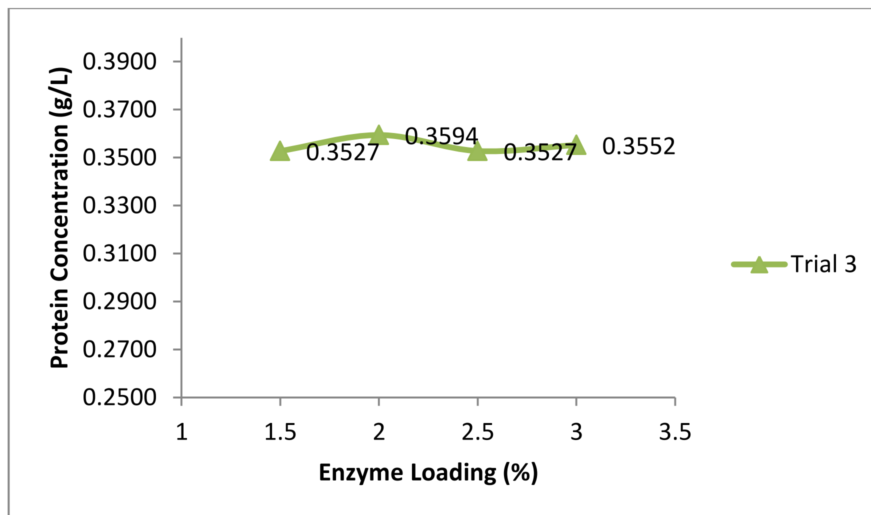

Figure 4-9 Protein concentration versus enzyme loading (%) of the sample solutions in third trial.

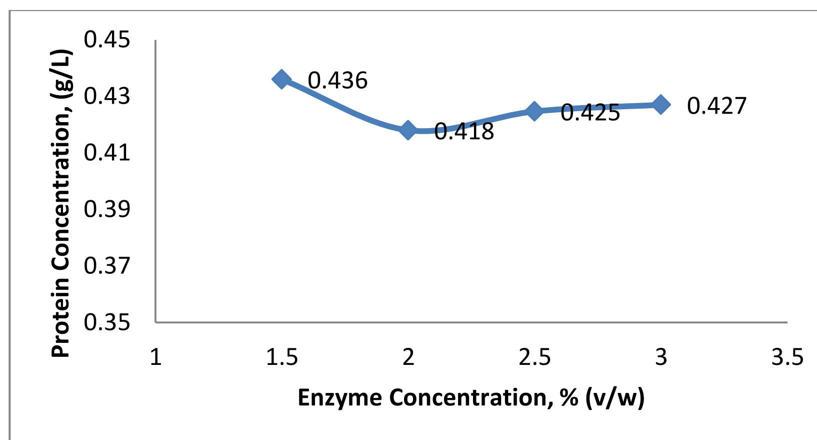

Figure 4-10 Average protein concentration versus enzyme loading (%) of the sample solutions for three trials.

The results that had been achieved shown that the concentration for the protein in the sample for all three replication of the sample solution means that the enzyme loading has been not really affected on the protein concentration yield. This statement was argued by (See, Hoo, & Babji, 2011) that the factors such as pH, time, temperature, and enzyme to substrate level will influence enzymatic activity and the yield of the product. However the enzyme loading will affect the enzyme activity which the higher the enzyme load will may decrease the hydrolysis activity.

All of the trials in this research on the variation of enzyme loading shows a less significant results where the protein in the sample is just at the same content with each another. However based on research conducted by See et al., (2011, the condition for the enzymatic hydrolysis proved to be 2.50% (v/w) for enzyme to substrate level, temperature of 55.30°C and pH of 8.39 is sure to be the optimum conditions to obtain the highest rate of enzymatic hydrolysis. Nevertheless, the optimum residence time and pH might affect each other with the same amount of enzyme.

The main factors that effect in the enzymatic hydrolysis of protein are the substrate concentration and quality, pre-treatment method applied, enzyme activity, and hydrolysis conditions such as temperature, pH, and mixing time. While for the optimum temperature and pH are functions of the raw material, the enzyme source, and hydrolysis duration (Taherzadeh & Karimi, 2007).One of the main factors that affect the yield and initial rate of enzymatic hydrolysis is substrate concentration in the slurry solution. High substrate concentration can cause substrate inhibition, which substantially lowers the hydrolysis rate. The extent of the inhibition depends on the ratio of total enzyme to total substrate (Sun and Cheng 2002). Problems in mixing and mass transfer also arise in working with high substrate concentration. The ratio of enzyme to substrate used is another factor in enzymatic hydrolysis. Obviously application of more protein, up to a certain level, increases the rate and yield of hydrolysis.

The analysis had been made by using Fourier Transform Infrared Spectroscopy to prove the results obtained. However, based on the analysis obtained from the FTIR analysis the results show the different between the absorbance of the amine group that shows that the protein contents in the solution. Figure 4-11 below shows the differences between the solutions analysed showing the results where the protein content is increasing with the increasing of the enzyme concentration in the sample. This is due to reason of the effect of enzyme concentration had made peptide bond cleavage to be higher even the time of hydrolysis is limited at only 1 hour in this study (See et al.,2011).

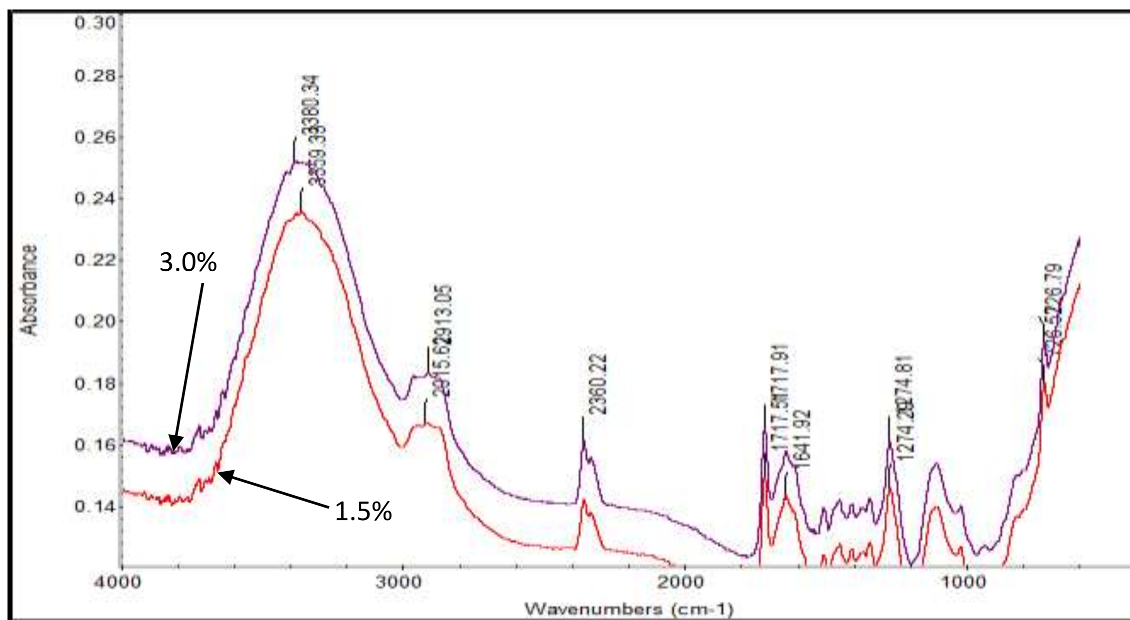

Figure 4-15 FTIR analysis on different enzyme loading of 1.5% (v/w) and 3.0 % (v/w)

4.6 CHEMICAL CHARACTERIZATION OF THE PROCESSED EBN BY FTIR

In Figure 4-13, FTIR spectrum of the processed EBN had been showed. It clearly reveals the major peaks associated with the carboxyl group. From the graph, it can be observed that O-H stretching vibration peak (ν = 3400-2400 cm^{-1}) of the carboxyl group can be found and also C-H broad alkyl stretching band (ν = 2850-3000 cm^{-1}) at the highest peak. The C=O stretch band found (ν = 1730-1700 cm^{-1}) indicate that the presence of the compound of carboxylic acid group in the molecular unit of protein. In the figure below the O-H stretching peak can be obviously be seen to be increased when the enzyme is it in the optimum condition. The enzymatic hydrolysis seems to be progressively increased the protein yield when the solution had been subjected to be changed from pH 7 to pH 8 to pH 10. This is because to extract or isolate the protein in a

successful manner, the use of either by using acid, saline or alkaline solution is needed (Ng et al., 2012). This had shown that the sample was hydrolysed by the aid of the protease enzyme used where the entire compound absent in the protein can be found highly in the processed sample with the optimum condition applied.

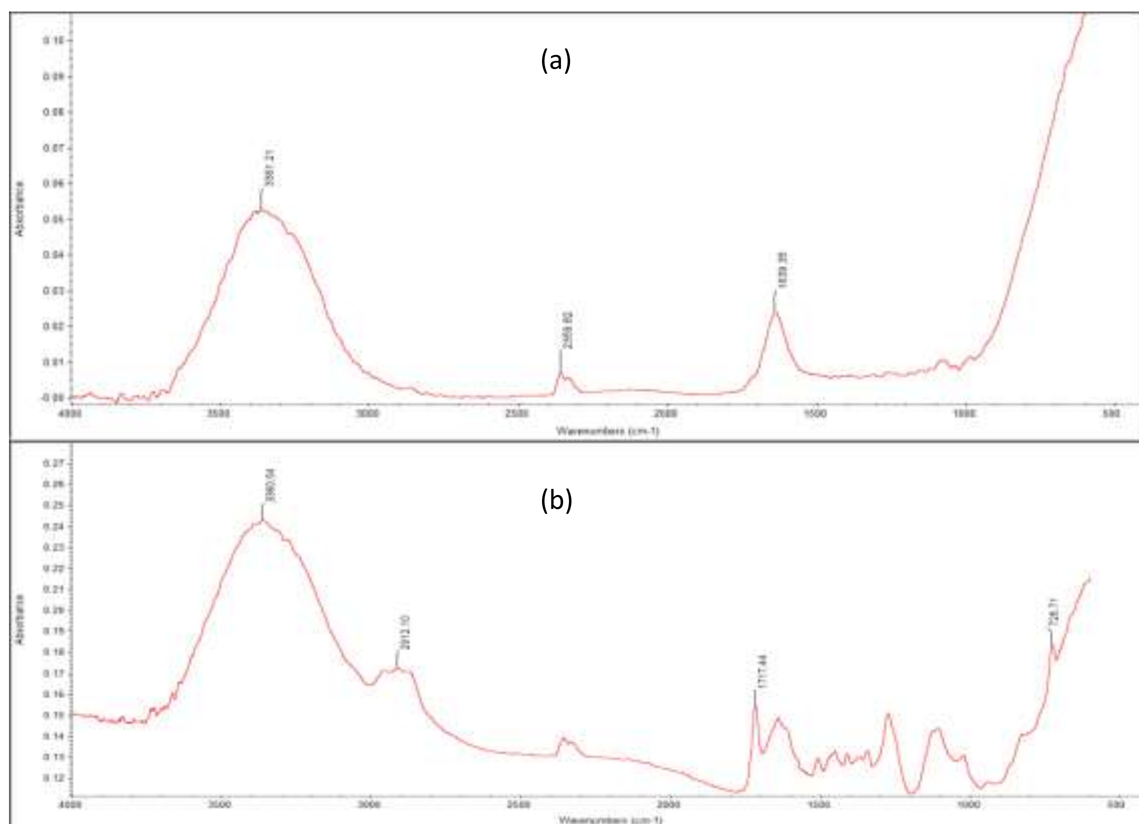

Figure 4-16 Comparison of analysis using FTIR from processed EBN sample from pH 7 (a) to pH 10 (b)

Based on the literature Ng et al. (2012) the structural repeat unit of proteins which called peptide group has 9 characteristic bands named amide A, B, I, II until VII. Amide I and amide II bands are two major bands of wavelength detected on the protein infrared spectrum. Amide I and amide II bands are two major bands of the protein infrared spectrum. The amide I band (ν = 1600 to 1700 cm^{-1}) can be associated with the

C=O stretching vibration in the group. Whereas for Amide II was the results from the N–H bending vibration and from the C-N stretching vibration. However the amide III band is usually weak in the FTIR spectroscopy but can be found in the region from ν =1250 to 1350 cm^{-1} (Ng et al., 2012)

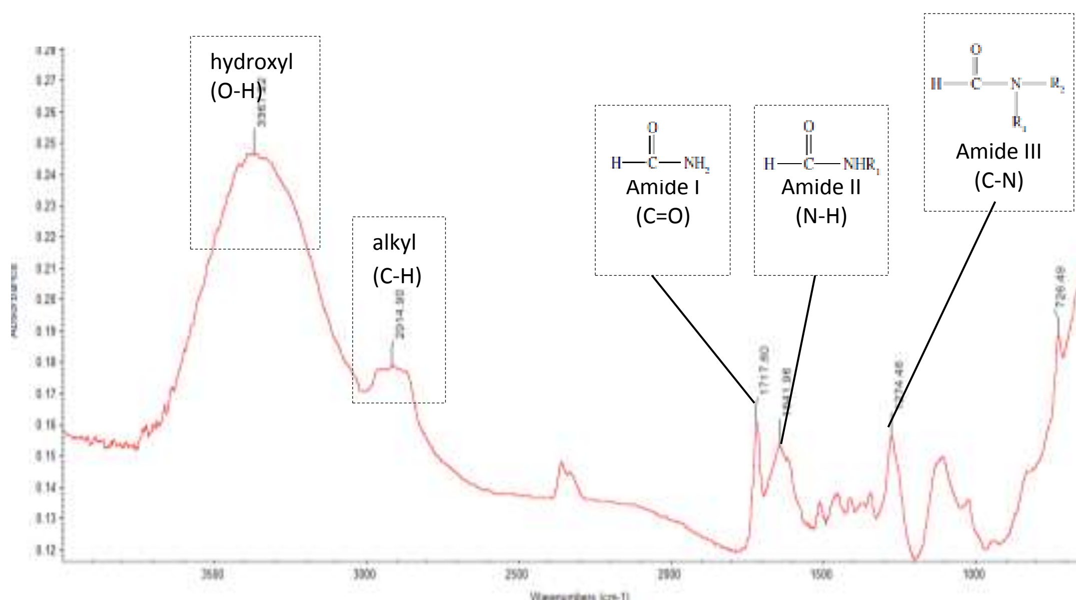

Figure 4-13 Available groups of compounds detected by on FTIR spectrum analysis on protein yield from processed EBN sample.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The effects of system variables such as the pH of the buffer solution, the concentrations of enzyme and substrates, and the reaction temperature were investigated during the enzymatic hydrolysis of protein from *Collocalia Fuciphaga* by using an alkaline buffered protease from *B. Licheniformis*. The temperature was fixed at 50°C which is the optimum temperature for the Alcalase enzyme. As expected, a high sensitivity with respect to pH was observed. An increase in the protein concentration when the pH increased until the optimum pH 8 to pH 9 was obtained which agreed the previous research results of pH 8.39 as the optimum value for the hydrolysis. However the results obtained from enzyme loading parameter shows insignificant results with the highest enzyme loading at 1.5% (v/w). The results opposed the hypothesis that the higher enzyme loading will exhibit a higher protein concentration where the value had should be at 2.5% (v/w).

5.2 RECOMMENDATION

A better adjustment for the experimental results to be obtained should be by increasing the parameter range, so that the significant result could be obtained to see the effect of the manipulation of the variables. These results produced from the research can be used as complementary information for modeling of similar degradation stage in the secondary stage hydrolysis for obtaining a better result. The other analysis method such as Scanning Electron Microscope (SEM) should be implement in order to give a view on the effect of enzyme on the sample structure.

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APPENDIX

Appendix 1: Preparation of Buffer Solution

1) Sodium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid per liter (0.2 M)

Solution B: 16.4 g sodium acetate per liter (0.2 M)

Referring to Table A-1 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 100 ml.

Table A-1: Preparation of 0.1 M Sodium Acetate Buffer

Desired pH	Solution A (ml)	Solution B (ml)
4.0	41.0	9.0
5.0	14.8	35.2

2) Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH2PO_4 per liter (0.2 M) Solution B: 45.6 g K_2HPO_4 per liter (0.2 M) Referring to Table A-2 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 200 ml.

Table A-2: Preparation of 0.1 M Potassium Phosphate Buffer

Desired pH	Solution A (ml)	Solution B (ml)
5.0	87.7	12.3
6.0	39.0	61.0
7.0	5.3	94.7

3) Glycine-NaOH buffer, 0.1 M

Solution A: 15.01 g glycine per liter (0.2 M)

Solution B: NaOH (0.2 M)

Referring to Table A-3 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 200 ml.

Table A-3: Preparation of 0.1 M Glycine-NaOH Buffer

Desired pH	Solution A (ml)	Solution B (ml)
9.0	50.0	8.8
10.0	50.0	32.0

Appendix B: Infrared Spectroscopy

Table B-1 IR Absorptions for Representative Functional Groups

Functional Group	Molecular Motion	Wavenumber (cm^{-1})
alkenes	=CH stretch	3100-3010
	C=C stretch (isolated)	1690-1630
	C=C stretch (conjugated)	1640-1610
	C-H in-plane bend	1430-1290
	C-H bend (monosubstituted)	~990 & ~910
alcohols	O-H stretch	~3650 or 3400-3300
	C-O stretch	1260-1000
aldehydes	C-H aldehyde stretch	~2850 & ~2750
	C=O stretch	~1725
ketones	C=O stretch	~1715
	C-C stretch	1300-1100
carboxylic acids	O-H stretch	3400-2400
	C=O stretch	1730-1700
	C-O stretch	1320-1210
	O-H bend	1440-1400
amines	N-H stretch (1 per N-H bond)	3500-3300
	N-H bend	1640-1500
	C-N Stretch (alkyl)	1200-1025
	C-N Stretch (aryl)	1360-1250
	N-H bend (oop)	~800
amides	N-H stretch	3500-3180
	C=O stretch	1680-1630
	N-H bend	1640-1550
	N-H bend (1°)	1570-1515

Appendix C : Amino acid Edible Bird's Nest extract

Table C-1 Amino acid distribution (mg/g) of edible bird's nest extract.

Name	Total amino acid	Flee amino acid	(F/T) x 100
Aspartic acid	40.44	0.08	0.19
Threonine	22.39	1.32	5.89
Serine	29.47	0.84	2.85
Glutamic acid	51.78	0.27	0.52
Proline	21.07	0	0
Glycine	18.34	1.77	9.65
Alanine	18.44	2.79	15.13
Cysteine	41.06	0.06	0.14
Valine	24.35	8.88	36.46
Methionine	5.77	5.07	87.86
Isoleucine	16.65	8.36	50.21
Leucine	26.06	14.19	54.45
Tyrosine	17.16	11.83	68.93
Phenylalanine	29.37	16.19	55.12
Histidine	16.54	5.26	31.80
Lysine	15.23	6.08	39.92
Arginine	18.36	4.78	26.03
Tryptophan	0	6.02	100

Appendix D : Lowry's Method

Table D.1 Reagents for Modified Lowry's Method

Reagent	Preparation procedure	Notes
A	Dissolve 20 g of sodium carbonate and 4 g of sodium hydroxide in 1 L distilled water	Keep refrigerated
B	Dissolve 2.5 g of copper sulfate ($CuSO_4.5H_2O$) and 5 g of sodium citrate in 1 L distilled water	Wrap the bottle with aluminium foil to avoid discolorization and keep refrigerated
Lowry solution	Mix reagent A and B in a 50:1 ratio	Prepare only when needed and keep refrigerated
Folin-Ciocalteu (stock 2.0 N)	Dilute the stock with distilled water in 1:1 ratio	Prepare only when needed

Modified Lowry's Method

1. Prepare Bovine Serum Albumin (BSA) at the concentration of 200, 500, 1000, 1500 and 2000 µg/ml.
2. Add 1 ml of Lowry reagent into 0.2 ml of each concentration of BSA, mix well and leave at room temperature.
3. After 10 min, add 0.1 ml of 1.0 N Folin-Ciocalteu reagent, mix well and leave at room temperature.
4. After 30 min, measure the optical density of the mixture at 750 nm against blank[1].
5. Plot the calibration curve (OD vs concentration) for each BSA concentration.
6. In order to determine the protein concentration in the sample, repeat the same procedure as described above (step 2-5) by replacing 0.2 ml of BSA solution with 0.2 ml of sample.

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[1] Replacing BSA with equal volume of distilled water, other reagents and steps are remained.