PRODUCTION AND CHARACTERIZATION OF PROTEIN EXTRACT FROM LEMPOYANG GINGER

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SUPERVISOR DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality of the award of the degree of Chemical Engineering (Biotechnology).

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I hereby declare that the work in this thesis is my own except for quotation and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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ABSTRACT

Antimicrobial protein has received tremendous attention especially by the pharmaceutical industry, which sources mainly extracted from nature such as herbs and plants. These sources are known for their nontoxic property, biodegradable, as well as easily available in the market. Among the numerous natural resources, the Lempoyang ginger which scientifically known as *Zingiber zerumbet L. Smith* had long known for its application as antifungal, anti-inflammatory, anti-ulceration and antioxidant. It is also known for treating diarrhea, coughs, asthma and some other skin diseases. Lempoyang ginger is selected for this research as very few information was published on protein of this *Zingiberaceae* family of which among all, zerumbone was widely extracted as the major component. This research aimed to produce the protein extract from Lempoyang using the expanded bed adsorption chromatography (EBAC) and then characterize it using the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The parameter that was varied for this research is the height of the bed settlement at 5cm, 6cm, 9cm and 11cm, with elution made in two-steps using 45% ethanol and 90% ethanol buffer solutions. Amberlite XAD7HP is used as adsorbents to bind the large complex protein molecules from Lempoyang feedstock with constant pump speed at 13rpm for each cycle. The eluted protein fractions are then subjected to Lowry's protein concentration determination technique before characterized for molecular weight determination using the SDS-PAGE tools. The dynamic binding capacity is determined from the 50% breakthrough of the initial feedstock solution, and the recovery ratio is determined for total protein obtained. From the results, protein concentration is higher when eluted with 45% ethanol buffer solution as protein were loosely bounded to the adsorbents while the first elution using 90% ethanol buffer solution showed a lower protein concentration. The washed protein and eluted protein fractions from EBAC which were tested on SDS-PAGE showed a protein band of eluted fraction protein band with molecular weight of 21.12 kDa.

ABSTRAK

Protein antimikrob telah menjadi tumpuan utama terutama kepada industri farmseutikal dimana sumber utamanya diperoleh secara pengekstrakan daripada tumbuh-tumbuhan dan herba. Sumber alam ini terkenal dengan ciri-ciri tidak toksik, kebolehuraian secara biologi, dan ia mudah diperoleh di pasaran. Diantara sumber yang mendapat focus adalah halia Lempoyang, dikenali dengan nama saintifiknya *Zingiber zerumbet L. Smith*, yang lama telah diketahui khasiatnya sebagai antifungi, anti-radang dan antioksida. Ia juga berupaya merawat diarrhea, batuk, asma dan beberapa masalah kulit lain. Halia Lempoyang dipilih untuk kajian ini kerana sedikit maklumat mengenai kajian berkaitan kumpulan *Zingiberaceae* dihasilkan, dan 'zerumbone' merupakan komponen protein utama yang diekstrak. Kajian ini bertujuan untuk menghasil dan mengkategori protein yang diekstrak dari Lempoyang menggunakan teknik penjerapan secara mengembang menggunakan Amberlite XAD7HP sebagai agen penjerap. Protein yang diperoleh kemudian dikategori menggunakan teknik SDS-PAGE selepas kepekatan protein diuji menggunakan teknik Lowry. Kapasiti jerapan dinamik diperoleh dari 50 peratus kemasukan sampel jus Lempoyang dan jumlah protein yang diperoleh kemudian dikira. Keputusan ujikaji menunjukkan perolehan dari 45 peratus etanol mempunyai kepekatan rotein lebih tinggi dari 90 peratus etanol kerana protein kurang terjerap pada Amberlite. Pecahan campuran protein dari basuhan dan elusi yang dikategorikan menggunakan SDS-PAGE menunjukkan sampel yang dielusi mempunyai berat molekul 21.12 kDa.

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LIST OF SYMBOLS

°C	Degree Celsius (temperature)
ϴ	Surface coverage
kDa	kilo Dalton
ml	Milliliter
cm	centimeter
nm	Nanometer
rpm	Revolution per minute
mg/ml	Milligram per milliliter
u	Superficial velocity
ε	Expanded voidage

LIST OF ABBREVIATIONS

EBAC	Expanded bed adsorption chromatography
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
GC-MS	Gas chromatography
CV	Column volume
OD	Optical density
BSA	Bovine serum albumin

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

1.1.1 Protein

The word ‘Protein’ was initially introduced by Jons Jacob Berzelius in 1838 from the Greek word (prota) which means “of primary importance” for large organic compounds with almost equivalent empirical formulas. Then, the protein study was made by James B. Sumner in 1926 showing that enzymes can be isolated and crystallized. The proof that protein has its specific structure was determined by Sir Frederick Sanger in 1955 by sequencing the first protein called insulin. Later in 1958, Max Perutz and Sir John Cowdery Kendrew came up with the three-dimensional structure of hemoglobin and myoglobin by X-ray diffraction analysis.

Proteins were classified accordingly to their functions such as enzymes, hormones, transport proteins, antibodies, receptors, signaling proteins, storage proteins etc. These compounds were also classified based on their locations in the living cells as well as the posttranslational modifications such as native proteins, cleaved proteins, prions and others. Proteins structural organization can be identified as primary, secondary, ternary and quaternary based on their folding and the protein interactions.

1.1.2 Antibacterial compound in *Zingiber zerumbet L. Smith*

An antifungal protein is one type of widely known protein that function to treat or destroy the activity of fungi which causes diseases or worst, fatalities in living cells such as plant and animal, including human. In spices such as the ginger plants namely the Zingiberaceae was widely used as medicine formulations for relieving stomachache, macerated in alcohol which was regarded as tonic and depurative. Zerumbone contains proteins functioning as antifungal agent, and was isolated from Zingiber Zerumbet (L) Smith having unique structure with a cross-conjugated ketone in an 11-membered ring displaying selectivity in cytotoxic characteristics towards cancer cell lines and normal cell lines. Throughout studies and experiments made using zerumbone, the compound showed an antiproliferative activity upon HepG2 cells as well as of non-malignant Chang Liver and MDBK cell lines (SA Sharifah Sakinah, S Tri Handayani and LP Azimahtol Hawariah, 2007). Zerumbone which also expressed as ZER was also found to suppress tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation is some potent manner. The compound was also indicated to be having distinct potentials for usage in anti-inflammation, chemoprevention and chemotherapy strategies (M. Akira et al., 2002).

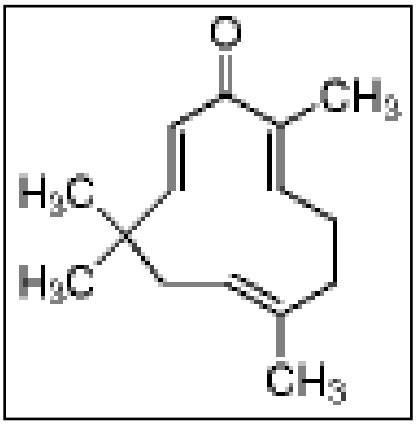

Figure 1.1: Zerumbone

1.1.3 Expanded Bed Adsorption Chromatography (EBAC)

Various methods were proposed and commercially used for protein production process including the liquid-liquid separation, packed bed adsorption, hydrodistillation and many others. Expanded bed adsorption (EBA) method was lately recognized as a tool used in advantage of yielding a higher concentrated product form the unclarified feedstock. It was considered an advantageous tool for this purpose as EBA process provide a less processing time and labor as it combines the clarification, initial purification and concentration step in one unit operation, hence reducing the cost of operations.

The EBA requires five (5) main steps which include the bed expansion stabilization, feedstock loading, washing of the bed column, elution with elution buffer, and finally regeneration of the bed. The bed needed to be stabilized before sample loading to determine the effective bed expansion ratio, and once sample is loaded into the column, the flowrate was to be measured in order to determine the effective loading flowrate. All these steps were carried out in an upwards direction as the adsorbents used for this research was Amberlite XAD7HP, which contain different sizes of molecules, and therefore, by applying the upwards motions, the bed expansion will be uniformly distributed accordingly to the sizes of the adsorbents. Also, this kind of load flow motion provided a better adsorption compared to packed bed adsorption.

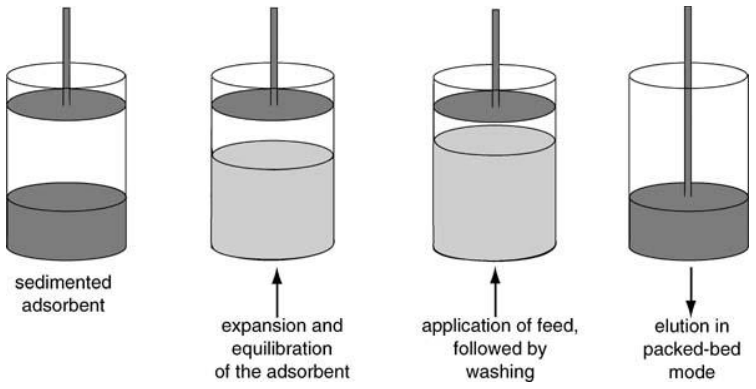

Figure 1.2: The expanded adsorption chromatography flow technique.

1.1.4 Adsorbents for Protein Adsorption: Amberlite XAD7HP

Amberlite XAD7HP was one type of adsorbent that showed capability of recovering plant extracts. Its large pores size was an advantage for adsorbing protein molecule in plant extracts or other natural sources compared to other adsorbents such as Amberlite XAD16 or Amberlite XAD1180 which might damage the peptides and protein molecules during adsorption. This acrylic polymer also showed ability to be performed in elution or regeneration step by using solvents, buffer or steam depending on the molecule type under consideration.

Amberlite XAD7HP was supplied in white insoluble beads, patented in macroreticular structure with aliphatic nature which able to adsorb non polar compounds from aqueous systems and also adsorbing the polar compounds from non-polar solvents. During shipment, this polymeric adsorbent was inhibited with NaCl and Na2CO3 salts to retard the bacterial growth. These salts were to be washed when the adsorbent was prior to use and the washing was suggested at a linear flowrate of 5-10m/h up to the required level.

1.2 PROBLEM STATEMENTS

The pharmaceutical industry has been developing methods to increase the production of antimicrobial protein from nature as it is seen as an advantage of using the biodegradable compounds which is non-toxic to human body and at the same times effective for treating diseases. Among all nature's products, the Lempoyang ginger has received tremendous attention in extracting and purifying the antimicrobial protein namely zerumbone mostly from the plant's rhizomes. However, due to very limited research being made on this plant, and very few information regarding this antimicrobial protein being published in journals, researchers has come out with various method for extracting this protein such as using the extraction and evaporation technique (2011), hydrodistillation (2009) as well as using the ion-exchange chromatography (2005).

1.3 OBJECTIVES

This research is purposely carried out in order to produce the protein extract from Lempoyang ginger, scientifically named *Zingiber zerumbet L. Smith*, by applying the expanded bed adsorption chromatography (EBAC) technique with Amberlite XAD7HP was selected as the adsorbents for this research. This research is also to characterize the protein extracted from EBAC according to their respective molecular weight by using the sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique, in order to determine the presence of antimicrobial protein namely zerumbone in the sample feedstock. At the end of the research, the main aim will focus on the parameters that can give a higher yield of protein concentration by varying the height of bed settlement and buffer concentrations.

1.4 SCOPES OF STUDY

The research is focused on the scopes as listed below in order to achieve the objectives outlined:

i) To study the extraction of protein from Lempoyang ginger using the expanded bed adsorption chromatography technique of Fastline 10 Column and Amberlite XAD7HP as the adsorbents.

ii) To determine the optimal settled bed height that will give a higher yield of protein from adsorption process of expanded bed adsorption chromatography technique (EBAC).

iii) To determine the presence of antimicrobial protein of zerumbone by characterization of eluted sample from expanded bed adsorption chromatography (EBAC) using the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique.

1.5 SIGNIFICANCE OF STUDY

This research applied the expanded bed adsorption chromatography (EBAC) technique for the purpose of protein extraction from unclarified feedstock. The uniqueness of this technique is that the feedstock loaded into the column is not necessarily be clarified as this EBA combines all three steps of feedstock clarification, concentration and initial purification within one unit operation. This combination is a major advantage among other techniques as it capable of reducing the cost of operation and production, reducing the time used as well as easy to be applied in industry.

Generally, protein concentration is determined using Lowry's method. However, the concentration value read is of total protein present in the sample. Hence, sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to characterize the respective protein accordingly to its individual molecular weight, which also seen as a more accurate method for determining the presence of desired protein in the sample.

CHAPTER 2

LITERATURE REVIEWS

2.1 *Zingiber zerumbet L. Smith*

Zingiber zerumbet (L) Smith is commonly known as wild ginger, and in most Asian countries; this ginger is called Lempoyang Ginger or "Shampoo Ginger". It is part of the Zingiberaceae family and originated from Indonesia which later distributed in Malaysia, Bangladesh, India, Nepal, Sri Lanka and other parts of the world including Hawaii. *Z. zerumbet* has its unique properties which act as an anti-inflammatory, anti-ulceration, anti-oxidant and anti-microbial agent, and it has been long used for medical treatment easing diarrhea, ear inflammation, swelling, sores, relieving rheumatic pain, etc. It usually can be found grown in the secondary forests or at the village edges and some also planted this ginger in gardens throughout the tropics. Zerumbone and α–caryophyllene are the major chemical compounds found in essential oil from the extraction of leaves and rhizomes of *Z. zerumbet*. The zerumbone oil after purified and processed was used as anticancer bioactive compound for treating cancers such as breast cancer and cervix cancer.

Figure 2.1: *Zingiber zerumbet (L.) Smith* plant.

2.2 ISOLATION AND PROTEIN PRODUCTION FROM *Z. zerumbet*

In expanded bed adsorption process, the adsorbents used need to be of size in range of 50 to 400 microns. The larger adsorbents were sedimented to the bottom of the column while the smaller particles at the top of the sediment. In extraction and purification of antifungal protein in Lempoyang Ginger, variant types of techniques were used such as Amberlite as adsorbent in Expanded Bed Adsorption (EBA) technique, Tris-HCl buffer which was applied to the DEAE-cellulose and affinity chromatography process, as well as using the hydrodistillation technique and modified Clevenger-type glass apparatus for extraction of essential oil with analysis of antifungal compound using GC-MS. However, only adsorption process showed a simpler purification technique compared to the others, hence, this technique was proposed to be applied in the protein purification industry especially the pharmaceutical industries which minimize the production cost due to single purification step of adsorption process.

2.3 ADSORPTION ISOTHERMS

2.3.1 Langmuir Adsorption Isotherm

The Adsorption of Adsorbate on Adsorbent surface isotherm was made by Irving Langmuir in 1916, and was awarded Nobel Prize in 1932 for the investigation of surface chemistry. The isotherm required three assumptions describing the adsorption of adsorbate to the surface of the adsorbents. First was that the adsorbent surface having contact to the solution containing adsorbate which was strongly attached to the adsorbent surface, secondly was the surface of the adsorbent having specific number of sites allowing adsorption of the solute molecules to the surface, and third was that the adsorption occurred in monolayer, which means the molecules attachments only occurred in one layer of adsorption (Duff, David G., Ross Sheina M. C. and Vaughan, D. Huw, 1988). The following chemical reactions represent the monolayer adsorption process which later, explain the isotherm.

$$\mathbf{A + S \leftrightarrow AS}$$

where AS represent the solute molecules bound to the surface site of S. Given the equilibrium constant consisting of the concentration of A and S expressed in units such as mol/cm2:

$$\mathbf{K} = \frac{[\mathbf{AS}]}{[\mathbf{A}][\mathbf{S}]} \quad (1)$$

The complete isotherm considered in terms of the surface coverage, Ɵ, was defined by the fraction of adsorption sites to which a solute molecule has attached to the surface. The unattached sites were expressed as (1- Ɵ) which related to the concentration by:

$$\frac{[\mathbf{AS}]}{[\mathbf{A}][\mathbf{S}]} = \frac{\boldsymbol{\theta}}{\mathbf{1}-\boldsymbol{\theta}} \quad (2)$$

The isotherm was expressed by replacing [A] as C, and the complete isotherm expression was achieved:

$$\mathbf{K_C} = \frac{\boldsymbol{\theta}}{\mathbf{C}(\mathbf{1}-\boldsymbol{\theta})} \quad \text{where} \quad \theta = \frac{\mathbf{K}}{\mathbf{1}-\mathbf{K}} \quad (3)$$

2.3.2 Freundlich Isotherm

The isotherm of Freundlich was proposed due to lower concentration of substance used, also as an alternative of determining the adsorption process developed by Herbert F. Freundlich. The Freundlich Isotherm was expressed as below, with k and n as parameters being empirically determined by plotting log Y vs. log C, using: Y = kC 1/n.

(Duff, David G., Ross Sheina M. C. and Vaughan, D. Huw, 1988).

2.4 EXPANDED BED ADSORPTION CHROMATOGRAPHY (EBAC) PROCESS

Expanded Bed Adsorption (EBA) represents the most exciting development in the field of bimolecular separations since the introduction of packed bed chromatography in the 1950s and it was applied for adsorption of streptomycin in 1950s and novobiocin in the 1970s (Richard J. P. Cannell). However, a stabilized expanded bed adsorption was developed in early 1990s with approach being used in processing of whole cell mammalian cell culture broth as well as successful in affinity chromatography application (John M. Walker & Ralph Rapley). Expanded bed adsorption was a one unit operation process accomplishing the removal of whole cell and cell debris (clarification step), concentration and initial purification of target protein from the crude extract loaded into the bed column (Michael John Lewis).

There are five main steps in adsorption process using the expanded bed technique. First was the expansion of packed bed into expanded bed using a particulate-free liquid. Secondly was the feeding of the sample solution containing particulate matter into the column until the adsorbent get saturated with the product. Then in third step, the column was upwashed as to remove the remaining particulate in the void of the bed. Fourth step was converting the expanded bed to packed bed state by gravity settling or reversed flow which product eluted from the adsorbents. Finally at fifth step of adsorption, the adsorbents were cleaned to remove the strongly bound impurities and re-equilibration procedure was made for next batch. Prior to 1980‟s, the expanded bed technique faced obstacles in determining the specific adsorbents being designed for the expanded bed procedures, which also the major obstacle for the technology to be implemented in both laboratory and industrial scale (Richard J. P. Cannell).

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The design methods for producing and characterizing the protein extract was initially made prior for carrying out the process. In the methods designed, all material, chemicals and equipments will be listed accordingly to the step of producing the protein extract using the expanded bed adsorption chromatography (EBAC) technique, and characterization of the protein using the sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique. The experiment was done in the bioprocess laboratory of Chemical Engineering in University Malaysia Pahang (UMP).

3.2 MATERIAL AND EQUIPMENTS

The main raw material used for this research project is the Lempoyang ginger rhizomes which were purchased from a market in Johor. The protein concentration determination will be using chemicals of Lowry's reagent, Folin-Ciocalteu reagent and the SDS-PAGE solutions.

3.2.1 Warring/Juice blender

The blender is used to separate the crude ginger juice from ginger biomass such as the ginger root fibers. The blender is of automatic setting, therefore the speed is kept constant for each juice extraction of ginger blending.

3.2.2 Vacuum filter

The vacuum filter was used to remove the remaining large solid particles in crude juice. A suspended-solid (SS) filter paper was used for the filtration as normal laboratory filter paper will take a longer time to produce an initial clarification of the crude juice.

Figure 3.1: Vacuum pump.

3.2.3 Expanded Bed Fastline 10 Column

The adsorption of protein compounds was carried out using the expanded bed of Fastline 10 Column at range of 5 to 12 cm of settled bed heights. Amberlite XAD7HP was used as adsorbents and the settled bed heights will be according to the settlement of this adsorbent in column.

3.2.4 UV-Vis Spectrophotometer

The UV-Vis spectrophotometer is used to determine the protein concentration of eluted fractions from EBAC. The ultraviolet wavelength determines the absorbance of protein present.

Figure 3.2: The UV-Vis spectrophotometer.

3.2.5 The SDS-PAGE Equipment

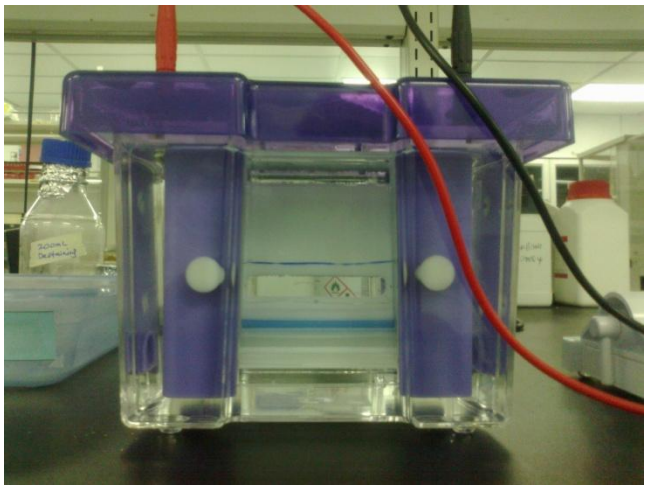

Figure 3.3: The SDS-PAGE tool.

3.3 RESEARCH METHODS

3.3.1 Ginger juice extraction

The ginger rhizomes were blended using a juice blender to obtain a pure crude ginger juice. 80ml of ginger juice was obtained from 200g of ginger rhizomes. It was then filtered using the vacuum filter and suspended solid (SS) filter paper to remove the large particles. The filtered juice was then divided into two portions at which one was reserved as unclarified feedstock while the other one was centrifuged at 5000rpm for 20 minutes as clarified feedstock for process optimization purpose. Ethanol buffer was also prepared by dilution to 35% ethanol, 45% ethanol and 90% ethanol from pure 95% ethanol.

Table 3.1: Dilutions of ethanol.

Vol. of ethanol 95% (ml)	Vol. of distilled water (ml)	Ethanol conc. (%)
35	65	35
45	55	45
90	10	90

3.3.2 Optimization of Binding, Bed Expansion and Elution Process

The optimization of binding and bed expansion of the bed column containing adsorbents was important as these steps will determine the efficiency of adsorption and affecting the flow rate of sample loading. The bed was initially filled with adsorbents, which in this research, Amberlite XAD7HP was used at an initial settled bed height of 5 cm. The base of the column was connected to the peristaltic pump via a tube and deionized water was left to flow in upward direction to observe the flow of fluid inside the column, as well as to observe any clogging occurred inside the base hole.

The speed of pump was set at constant rotation of 13rpm and the Amberlite expansion was observed to determine the bed expansion. This step was repeated few times in order to get a constant bed expansion ratio before loading the sample. Once the expansion height stabilized, the unclarified feedstock was loaded into the column and the flow rate was recorded.

The optimization of elution was made by first testing the clarified feedstock to the expanded bed column along with the loading of buffer solution and elution buffer with bed filled with Amberlite XAD7HP. The efficiency of the elution step and recovery of the protein was tested by measuring the purification factor by calculating the concentration of total protein in the feedstock, after binding and after elution step.

3.3.3 Expansion Characteristics of Adsorbent

At 25°C of normal room temperature, the expansion characteristics of the adsorbents in column were investigated. The adsorbent bed was expanded using the ethanol buffer which was used for adsorption process, with increase of superficial velocity. The degree of bed expansion corresponding to each velocity was recorded and expressed as a ratio of the expanded height, H to the settled bed height of adsorbents, H_0. The data were then fitted with the Richardson-Zaki correlation:

$$u = u_t \varepsilon^n \quad (4)$$

where u is the superficial velocity, u_t is the terminal settling velocity, ε is the expanded voidage, and, n is the bed expansion index. The settled bed voidage, ε_0 was assumed to have the value of 0.4 (Tong et al, 2001).

3.3.4 Dynamic Binding Capacity

The determination of breakthrough capacity provided a more accurate measurement of total expanded bed function as well as the impact on the protein binding capacity, by means of chromatographic function of the system (Pharmacia Biotech).

The purpose of determining the breakthrough capacity was mainly to verify that the process yielded a stable expanded bed providing consistency in breakthrough capacity when applied by scales. Initially, the breakthrough was carried out with BSA and the binding was measured by calculating the applied volume of BSA solution per ml of adsorbent.

In another method of determining the dynamic binding capacity breakthrough, which was applied in this research experiment was that, the plotting of concentration protein at eluted solution per inlet (C/C_O) against volume of feedstock loaded into the expanded bed column, was recorded and from the plotted graph, the breakthrough was determined at 50% of feedstock solution being loaded. According to the method used by F. C. Chong et al. (2009), the dynamic binding capacity, Q_B (mg of protein adsorbed per ml of settled adsorbent) was calculated using the following formula:

$$Q_B = \frac{C_0 V_b}{V_s} \tag{5}$$

where C_0 is the initial concentration of the feedstock (mg/ml), V_b is the volume at 100% breakthrough (ml) and V_s is the settled volume of the adsorbent (ml).

3.4 ANALYTICAL METHODS FOR PROTEIN DETERMINATION

3.4.1 Lowry's Method

The protein extract obtained in fractions of elution of EBAC was introduced to Lowry's reagent to determine the total protein concentration. The bovine serum albumin (BSA) was used as the standard with concentrations made varied and read on 750nm using the UV-Vis spectrophotometer. A graph plotted with absorbance value (nm) versus the BSA standard concentration (mg/ml) was used to determine the protein concentration in eluted fractions according to their respective absorbance values.

3.5 BATCH ADSORPTION OF FEEDSTOCK IN EBAC

3.5.1 Feedstock Solution Loading

Two extract solutions of unclarified feedstock and clarified feedstock were prepared with initial adsorption process being carried out using the clarified feedstock. Before the sample was loaded, Amberlite was brought to contact to the binding buffer which in this case, the binding buffer was 35% ethanol loaded in upward direction, causing bed of adsorbent to expand to the stabilized height as such in the optimization using deionized water. Once the buffer has all used up, sample solution of clarified feedstock was loaded in the same manner and for each 5 ml load, the time was recorded as to determine the flow rate of sample loading.

Figure 3.4: Feedstock solution of unclarified and clarified juice.

3.5.2 Washing

Once the feedstock solution has finished, the washing buffer was loaded into the column at 5 CV of volume sample loaded. The washing buffer used will be of same concentration as the binding buffer.

This step of washing was to remove the impurities inside the column as well as removing the unbounded protein molecules from the fluidized solution. The solution obtained will be tested for its concentration as to determine the ratio of unbounded protein from the system.

3.5.3 Two-Step Elution

After washing, the bed has now ready for elution process. Two elution buffer solutions were used for this research experiment, which were 45% ethanol for the first step elution, and 90% ethanol for the second elution. The first elution of 45% ethanol will remove the remaining impurities and also, remove the loosely bound protein in the expanded solution inside the column. Once the buffer has finished, the second elution buffer of 90% ethanol was loaded in upwards manner with flow rate being recorded for each 10 ml loading. Periodically by 10 ml of eluted product collection, the time was recorded in order to determine the flow rate of elution of product from the column. These fractions will be measured for their concentration to determine the yield of protein and each of them will also be measured for their molecular size on SDS-PAGE.

3.5.4 Re-equilibration

Re-equilibration was carried out for the expanded bed column once elution has completed. In order to re-equilibrate or regenerate the system, the peristaltic pump was stopped and the elution buffer was substituted to the regeneration buffer, and expansion was carried out once again. After this step has finished, the adsorption can be carried out for the unclarified feedstock by applying the same procedure as such made for the clarified juice.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Protein Extraction using Expanded Bed Adsorption Chromatography (EBAC) and adsorbents Amberlite XAD7HP

The adsorption process is using the Fastline 10 Column expanded bed that has maximum bed height of 30cm and column diameter 10mm. The sample loaded into the column is made constant per batch adsorption at 5ml with variant of adsorbents settled in bed. This settlement is the parameter used to measure the amount of protein adsorbed during the adsorption process, of which four (4) settled bed heights were tested, which includes 5cm, 7cm, 9cm and 11cm. The amount of adsorbents loaded into the column is calculated by using the density of Amberlite XAD7HP at 1.060g/cm^3 and the table below showed the amount of adsorbents used per bed height by measuring the its volume in column:

$$\text{Area of column} = \pi\,(0.5\text{cm}) = 0.7854\ \text{cm}^2 \qquad (6)$$

Table 4.1: Amount of Amberlite XAD7HP in settled bed column.

Height of adsorbent in column (cm)	**5**	**6**	**9**	**11**
Volume of adsorbents in bed (cm^3)	3.927	4.712	7.069	8.639
Amount of Amberlite XAD7HP (g)	4.163	4.995	7.493	9.158

4.2 Protein Extract Concentration Determination from Expanded Bed Adsorption Chromatography (EBAC) using Lowry's Method

The adsorbed protein in column is eluted from the expanded bed column using 90% ethanol of which 10ml of each fraction being collected to determine the elution flowrate. The BSA solution was prepared and the optical density (OD) value for the standard was plotted as in the graph below:

Table 4.2: Bovine Serum Albumin (BSA) concentrations for standard curve.

BSA (µg/mL)	0	200	500	1000	1500	2000	2500	3000	3500
OD (nm)	0	0.142	1.153	1.487	1.848	2.086	2.206	2.502	3.002

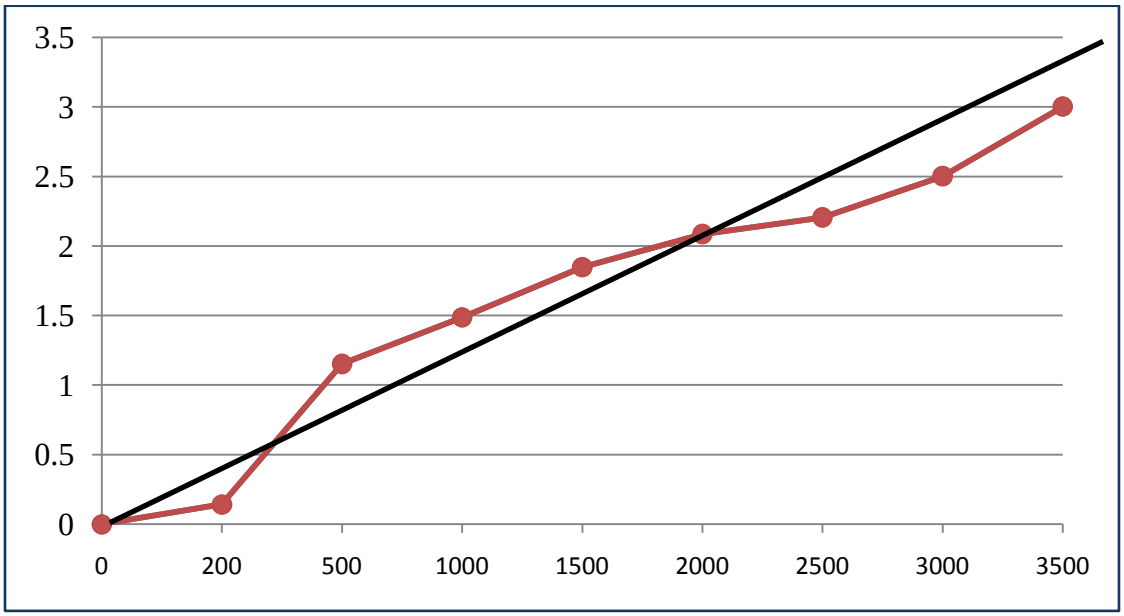

Figure 4.1: BSA Standard Solution curve.

The eluted protein fractions from the expanded bed column were then subjected to the same principle of Lowry's method which was used for BSA concentration determination. The optical density (OD) values for these fractions were listed in table below:

Table 4.3: The optical density (OD) values for the eluted fractions from EBAC.

Fraction	OD value (nm) for sedimented bed			
	5cm	6cm	9cm	11cm
Feed	3.0	3.0	3.0	3.0
Wash 1	0.566	0.840	1.320	1.856
Wash 2	1.325	1.516	1.892	2.124
Elute 1	1.164	1.205	1.246	1.287
Elute 2	0.948	0.957	1.198	1.258
Elute 3	0.917	0.930	0.983	1.045

Table 4.4: The protein concentration value of eluted protein fractions.

Fraction	Concentration Protein (mg/mL) for sedimented bed			
	5cm	6cm	9cm	11cm
Feed Sample	3.25	3.25	3.25	3.25
Wash 1	0.30	0.46	1.10	1.75
Wash 2	1.10	1.35	1.81	2.10
Elute 1	0.85	0.92	1.00	1.05
Elute 2	0.60	0.63	0.91	1.00
Elute 3	0.56	0.59	0.68	0.76

The fraction values obtained from absorbance values above were as average as both the washing and elution came from one time loading, therefore, the yield will be easier to be determined:

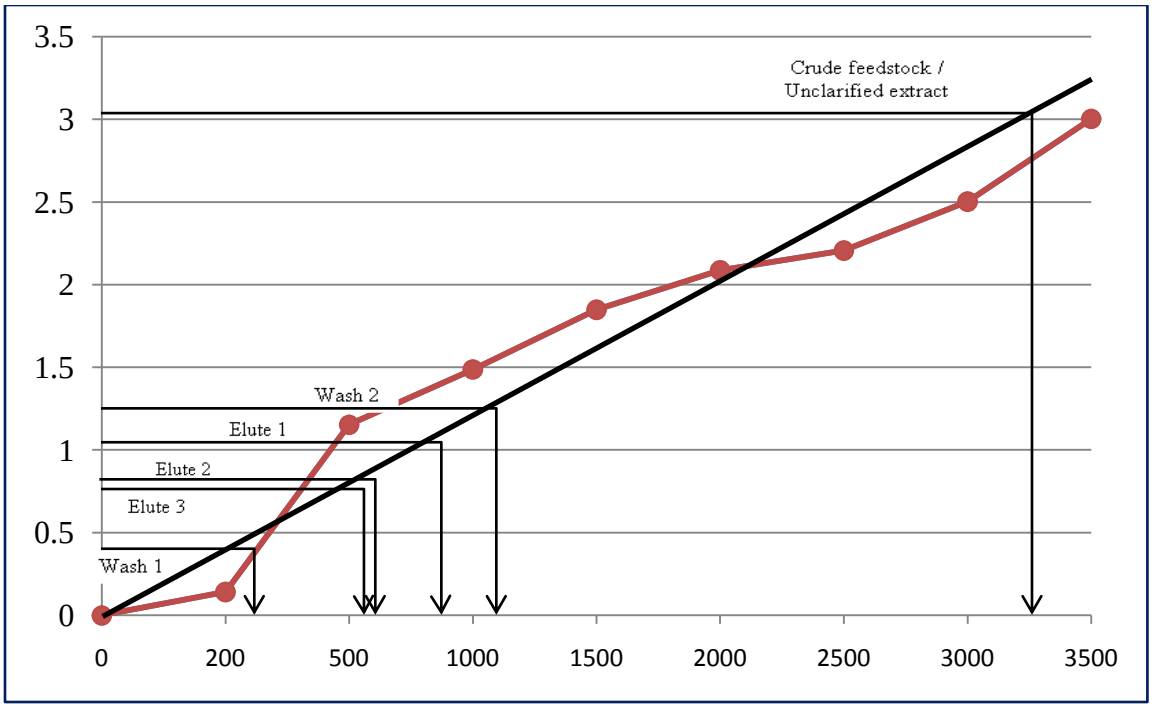

Figure 4.2: The OD-concentration plot for sedimented bed height 5cm.

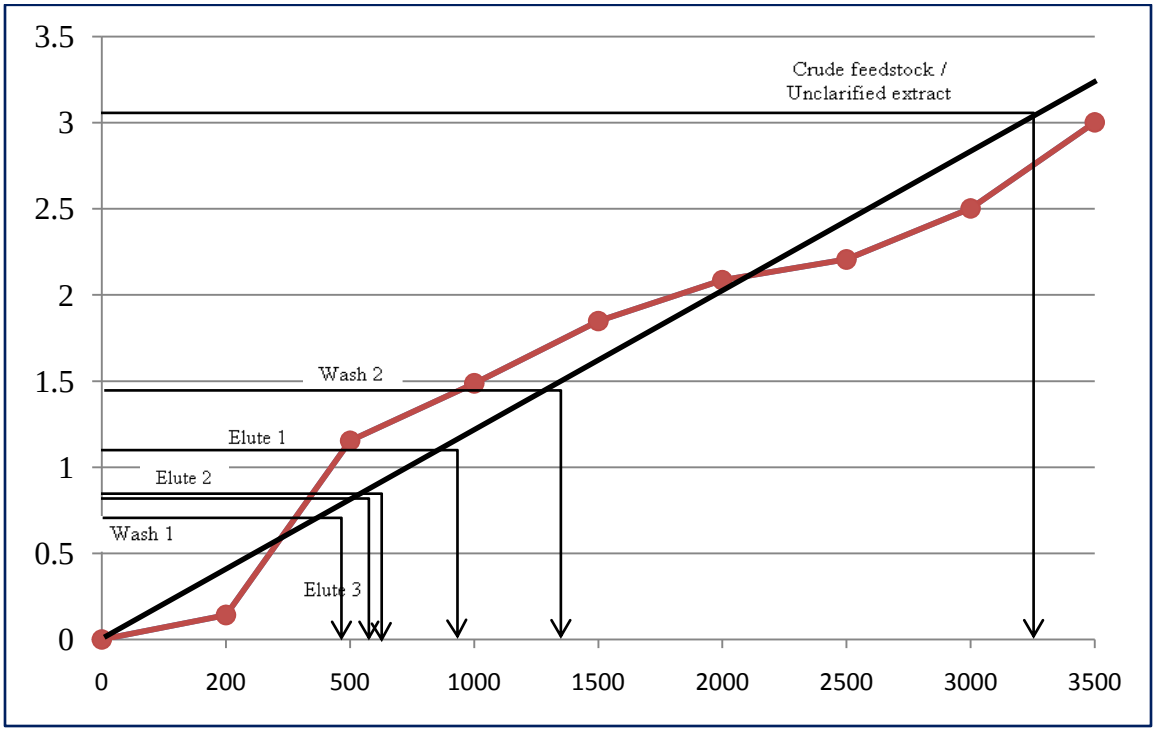

Figure 4.3: The OD-concentration plot for sedimented bed height 6cm.

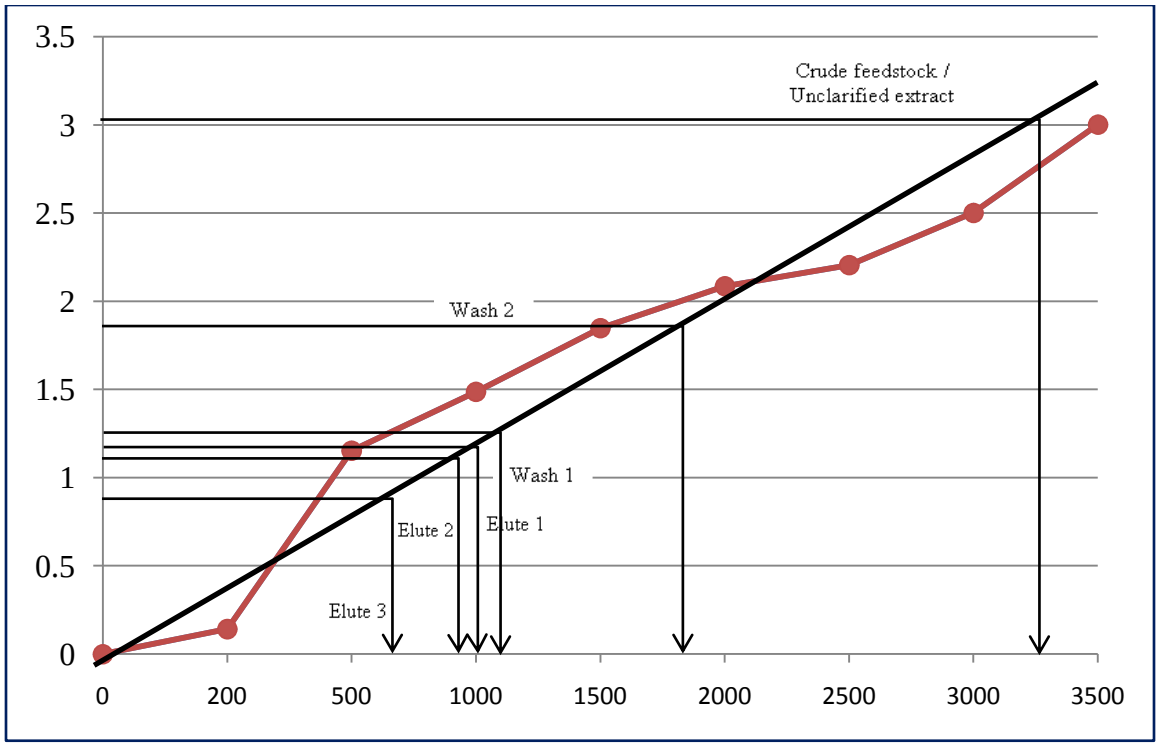

Figure 4.4: The OD-concentration plot for sedimented bed height 9cm.

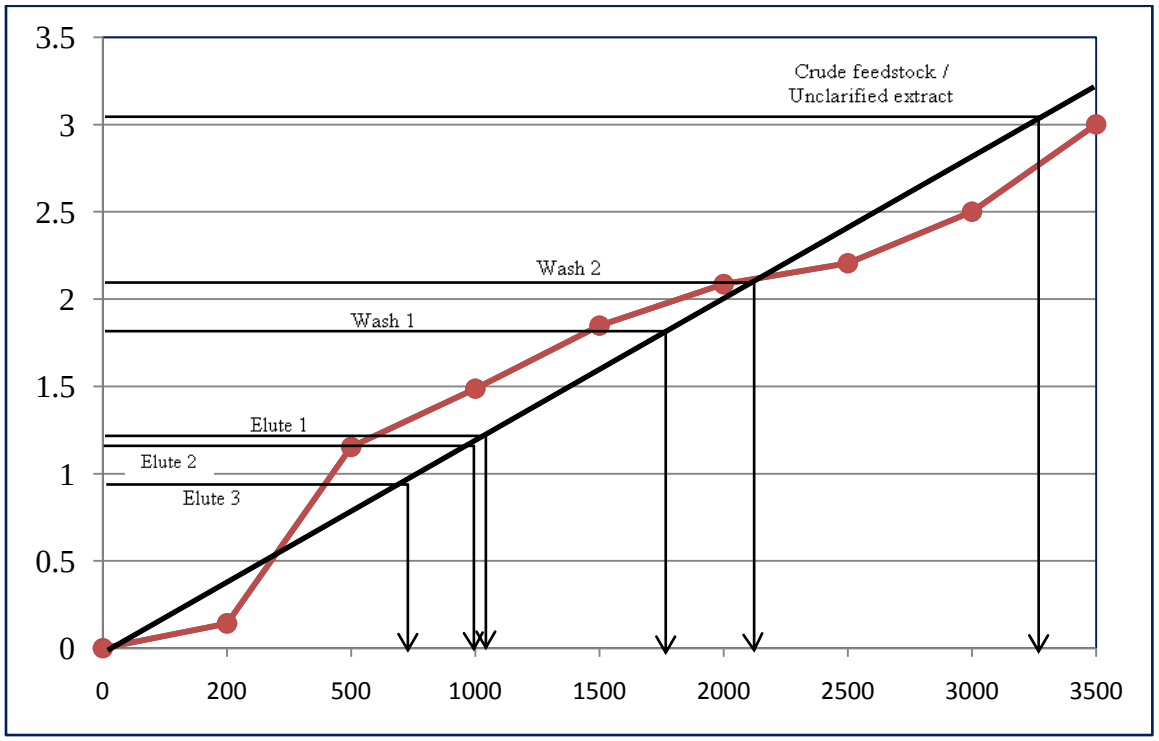

Figure 4.5: The OD-concentration plot for sedimented bed height 11cm.

Table 4.5: Average protein concentration values for 5cm, 6cm, 9cm and 11cm.

Fraction	Run 1 (nm)	Run 2 (nm)	Run 3 (nm)	Run 4 (nm)
Feed Sample	3.25	3.25	3.25	3.25
Washing	0.70	0.91	1.46	1.93
Elution	0.67	0.71	0.86	0.94

From all four graphs plotted as in Table 4.2, Table 4.3, Table 4.4 and Table 4.5, the eluted protein fractions give a lower protein concentration value compared to the washing fractions, except for run 4 where the eluted protein fractions has a higher concentration read. The phenomenon of this different protein concentration is because, during washing, the unbounded and loosely bounded proteins that fail to bind to the adsorbents in column were washed from the bed, and upon collection, these fractions contain a higher amount of protein from the feed. Washing buffer used for this step is of same concentration as in binding buffer loaded earlier, therefore, during this washing stage, the protein that has bind to the surface of the adsorbents will remain attached.

The elution buffer of ethanol at concentration 45% in the first elution step, is to remove the loosely bounded proteins that did not form a strong polar bind to the adsorbents surface, therefore, in elution 1 of each run, the absorbance reading is still higher as the ethanol concentration is slightly increased from the washing buffer. However, during elution of which 90% ethanol concentration is used, the protein that bind strongly to the adsorbents surface was detached and desorption occur in this stage. The next two eluted fractions give a decreasing value since the protein that remains in the column is the one that has a higher binding affinity, that once a higher buffer concentration is used, this binding to the adsorbents surface is completely broken.

In comparison to all four plotted graphs, the trends of protein concentration showed that the higher bed settlement of run 1 and run 2 gives a higher protein concentration compared to run 3 and run 4 which has a lower bed settlement. It is believed that when the adsorbents in bed

expand at a higher level, the time for proteins in feed sample to bind to the adsorbents surface increased which results in a higher protein concentration eluted from the column.

The yield of total protein eluted from the expanded bed adsorption chromatography is then calculated using the following formulas as well as the recovery from the feedstock of ginger juice by means of its concentration value:

$$Yield = \frac{concentration \quad protein \quad in \ eluted \quad fraction}{concentration \quad of \ juice \ in \ feedstock} \text{ x } 100\% \qquad (7)$$

Table 4.6: Percentage of protein yield values.

Fraction	**Protein Yield (%)**			
	5cm	**6cm**	**9cm**	**11cm**
Washing	21.54	27.85	44.77	59.23
Elution	20.62	21.94	26.55	28.83

The table above showed that the recovery ratio of eluted fraction was higher at sedimented bed height 11cm with a total of 28.83% of protein recovered from the adsorption process. The wash fractions for bed sediment at 9cm and 11cm showed a higher protein concentration being removed from the column is generally because the protein loaded failed to bind and form polarity bond to the adsorbents, or probably because the surface area of the adsorbents did not suit the type of protein present in the feedstock.

4.3 Bed expansion characteristics

Table 4.7: The bed expansion ratio and loading flowrate.

FRACTION	RUN 1	RUN 2	RUN 3	RUN 4
Sedimented Bed Height, H_0 (cm)	5.0	6.0	9.0	11.0
Expanded Bed, H (cm)	8.7	10.6	17.8	21.6
Expansion Ratio (H/H_0)	1.740	1.767	1.935	1.965
Loading flow rate (cm/h)	108.295	108.663	115.536	115.859

From the expansion ratio and flowrate of each running experiments, the superficial velocity is determined using equation v= Q/A where v is the superficial velocity, Q is the loading flowrate and A is the cross sectional area of the column.

Table 4.8: Superficial velocity for loading feedstock.

Sedimented bed height (cm)	Flowrate, Q (ml/s)	Flowrate, Q (cm^3/h)	Superficial velocity, v (cm/h)
5	0.098	352.8	449.198
6	0.099	356.4	453.782
9	0.119	428.4	545.455
11	0.120	432.0	550.038

4.4 Dynamic Binding Capacity

The dynamic binding capacity was measured by the 50% breakthrough of feedstock from initial concentration value, of which in this case, the initial protein concentration is 3.25 mg/ml, and focusing on the selected flowrate of 115.859cm/h for sedimented bed height 11cm, the 50% breakthrough is after 26.5ml of feedstock eluted from the column. The binding of protein to adsorbents for each sedimented bed height was also calculated using equation (5).

Table 4.9: Binding capacity of protein to adsorbents.

Sedimented Bed Height (cm), V_S	10% breakthrough, V_b (ml)	Binding capacity, Q_b (mg/mg adsorbent)
5	1	0.65
6	1.5	0.813
9	2	0.722
11	2.5	0.789

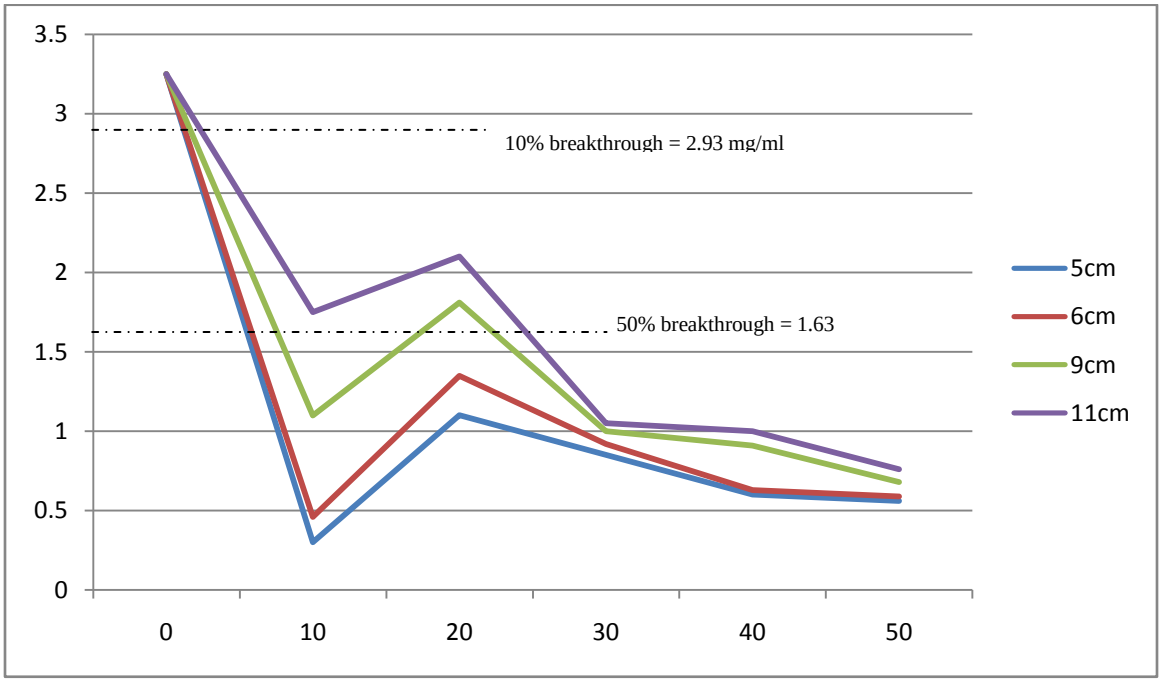

Figure 4.6: Expansion characteristic for determination of 50% breakthrough of feedstock solution and 10% breakthrough for dynamic binding capacity.

4.5 Protein Characterization by Sodium-Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein extract in eluted fraction from expanded bed adsorption chromatography (EBAC) is introduced to the SDS-PAGE for characterization process. The unknown protein from the sample which forms a clear blue band is determined for its size in Dalton, by measuring the distance of that unknown protein travelled from the top of the gel. The protein marker which consists of ten (10) protein bands is used as a standard for this process. Since the protein extract from EBAC technique consist of a number of macromolecules, the SDS-PAGE technique is used to separate the complex protein into subunits of which in this manner, the individual protein can be characterized and its molecular mass can be calculated by using the formula below in order to find the R_f value of the unknown protein of the sample.

$$R_f = \frac{distance\ travelled\ by\ band\ from\ gel\ top}{total\ length\ of\ gel} \quad (8)$$

The R_f values of the protein marker are then calculated and a graph is plotted with the log of molecule size versus R_f values. From this graph, the unknown protein which earlier, had its R_f value determined, is plotted and the log value of molecule size is then determined which also gives the molecular size of the unknown protein in the sample.

From the protein sample introduced to the SDS-PAGE, the unknown protein band formed is in between the seventh and eighth band of the protein marker, and the R_f value is calculated using the formula below:

$$R_f = \frac{4.8\ \text{cm}}{14.2\ \text{cm}} = 0.338$$

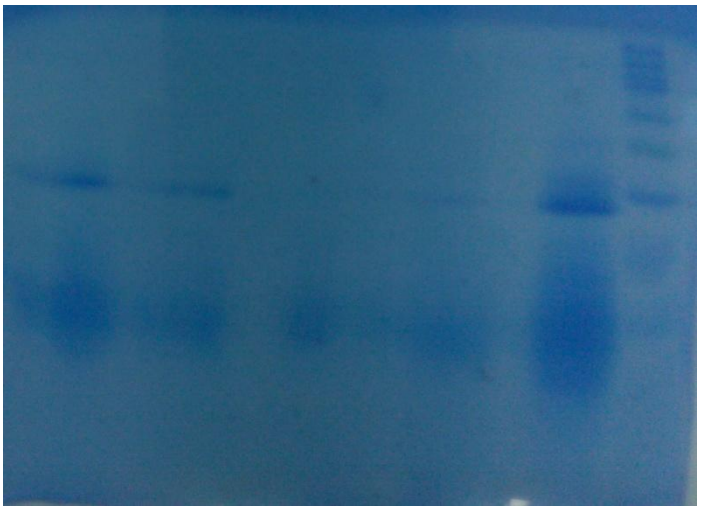

Figure 4.7: The SDS-PAGE gel for eluted protein fraction with the right-most figure is the protein marker band and the others are the sample from washing and elution from EBAC.

Table 4.10: The R_f values of protein marker.

Size (Dalton)	Log	Distance (cm)	R_f
250,000	5.3979	0.5	0.0352
150,000	5.1761	0.9	0.0634
100,000	5.0000	1.2	0.0845
75,000	4.8751	1.5	0.1056
50,000	4.6990	2.2	0.1549
37,000	4.5682	3.1	0.2183
25,000	4.3979	4.5	0.3169
20,000	4.3010	6.5	0.4577
15,000	4.1761	8.3	0.5845

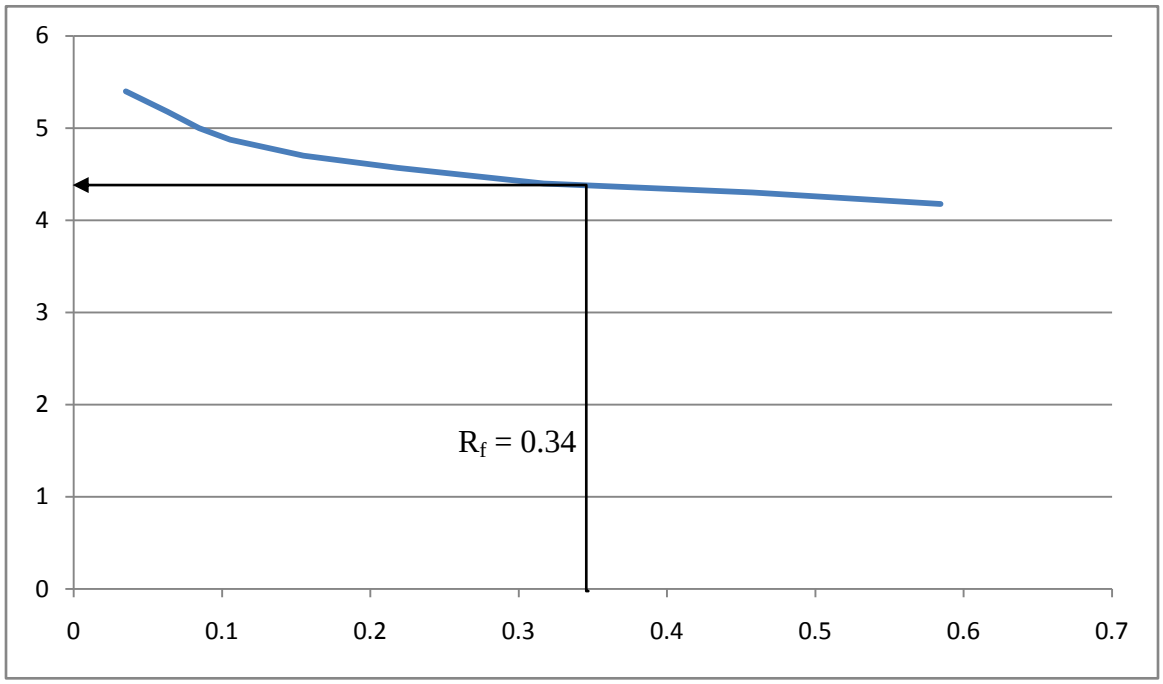

Figure 4.8: Graph of log kDa versus R_f.

This R_f value is plotted on the standard curve of the protein marker, of which the log value of molecular size of the unknown protein is 25,118.86 Daltons or 21.12kDa.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

In conclusion to this research project that implies the expanded bed adsorption chromatography (EBAC) and Amberlite XAD7HP as adsorbents with variant of settled bed height showed that a higher bed settlement of adsorbents will give a higher protein concentration yield by applying the two-step elution technique. This two-step elution technique of different ethanol buffer concentration is seen as an efficient method to produce the protein extract from unclarified Lempoyang ginger feedstock as the first elution using 45% ethanol buffer removes the protein molecules that do not fit to the pore size on adsorbent surface as well as protein that are loosely bound to the adsorbents by washing it in the first elution stage, removing the impurities from the column as well. The 90% ethanol buffer concentration showed that a higher concentration of total protein yield can be obtained as a more concentrated ethanol buffer will detach the affinity binding of protein and adsorbents, and desorbs them from the streamline with an optimum flowrate at 115.7 cm/h.

5.2 RECOMMENDATIONS

The determination of protein concentration using Lowry's method is seen as ineffective as the main aim for industrial product will be the antimicrobial protein of zerumbone, therefore, a standard identifying method to determine the concentration of this protein should be developed or, with a zerumbone standard, the eluted protein from the expanded bed column can be specifically determined and its concentration value can also be obtained using the HPLC technique. The effectiveness of the protein extracted and eluted from the feedstock using the EBAC can also be enhanced by separating the ethanol buffer and the protein using ethanol evaporation technique of which a more concentrated protein will be recovered at the end of the process and this protein is subjected to antimicrobial assay for observing the antimicrobial activity carried out. The adsorption of the respective protein can also be increased by varying the ethanol concentration for binding buffer as to increase the polarity of adsorbent surface to increase the affinity binding of proteins, which then increase the concentration of the protein eluted from the column. However, in order to observe the efficiency of which ethanol buffer yield a more higher protein concentration, a separate elution buffer can be used by applying a one-step elution process instead of using the two-step elution buffer.

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APPENDIX A

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.

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

APPENDIX B

Sodium Dodecyl Sulfate Polycryamide Gel Electrophoresis (SDS-PAGE)

1. Prepare stock and working solutions as shown in Appendix A4 and A5.
2. Clean and assemble vertical slab gel casting unit.
3. Prepare separating gel by mixing the solutions as indicated in Appendix A6. Always prepare fresh and add TEMED last.
4. Immediately, pour about 5.8 mL of the solution into each side of the casting apparatus.
5. Allow to polymerize at room temperature.
6. Once the separating gel had polymerized, prepare the stacking gel. Always prepare fresh and add TEMED last.
7. Pour 1.8 mL of stacking gel onto the top of the separating gel at each side.
8. Insert comb to make wells for sample loading.
9. Allow stacking gel to polymerize at room temperature.
10. After polymerization, detach the casting base and place the slab in the chamber of electrophoresis system that is filled with electrophoresis buffer.
11. Pull the comb slowly out from the gel.
12. Dissolve sample in either reducing or non-reducing sample buffer (refer Appendix A.4) in a ratio of 1:1 or greater (depending on their concentration) on the basis of their volume.
13. Heat reducing samples for 5 min at 98°C and fast spin for 1 second prior to electrophoresis. Do not heat non-reducing sample.
14. Load samples and molecular weight marker into the wells at the volume of 15 µL/well.
15. Conduct the electrophoresis at a voltage of 150 V for about 60 min.
16. Turn off power supply and remove electrode plugs.
17. Remove gels and place in a small container with small quantity of 50% Methanol.
18. Let the gel stand for 1 hr with gentle shaking.
19. Transfer gel to container containing about 20 ml staining solution.

20. Agitate gently for 2 hr on an orbital shaker. Cover the container.

21. Remove staining solution and rinse the gel with a few changes of water.

22. Add destaining solution and agitate for ½ hr.

23. Change destaining solution and agitate overnight.

24. Take photo of gel using Bio-imaging system.

25. Use the setting: transluminating → white light; reflecting → white light.

B1. Stock solutions required for SDS-PAGE

Solutions	Volume (mL)	Concentration	pH
Tris-HCl	100	2 M	8.8
Tris-HCl	100	1 M	6.8
Sodium dodecyl sulfate (SDS)	100	10% w/v	-
Glycerol	100	50% w/v	-
Bromophenol blue	10	1% w/v	-

B2. Working solutions required for SDS-PAGE

Solutions	Formula	Volume (mL)
A	75 mL 40% (w/v) acrylamide 25 mL distilled water	100
B	75 mL 2 M Tris-HCl (pH 8.8) 4 mL 10% (w/v) SDS 21 mL distilled water	100
C	50 mL 1 M Tris-HCl (pH 6.8) 4 mL 10% (w/v) SDS 46 mL distilled water	100
10% (w/v) ammonium persulfate	0.5 g ammonium persulfate 5 mL distilled water	5
Electrophoresis buffer	6 g Tris 28.8 g glycine 2 g SDS in 2 L distilled water (pH ~8.3)	2000
Sample buffer	0.6 mL 1 M Tris-HCl (pH 6.8) 5 mL 50% glycerol 2 mL 10% SDS 0.5 mL 2-mercaptoethanol 1 mL 1% bromophenol blue 0.9 mL distilled water ***Note: Omit 2-mercaptoethanol when preparing non-reducing sample buffer***	10

Staining solution (Coomassie stain)	1 g Coomassie Blue R-250 450 mL methanol 450 mL distilled water 100 mL glacial acetic acid	1000
De-staining solution (Coomassie stain)	100 mL methanol 100 mL glacial acetic acid 800 mL distilled water	1000

B3. Volume of various stock solutions required to prepare the separating and stacking gels

Solutions	**10% Separating gel**	**5% Stacking gel**
A	5 mL	0.67 mL
B	3.75 mL	-
C	-	1 mL
Distilled water	6.25 mL	2.3 mL
Ammonium persulfate	75 µL	30 µL
TEMED	25 µL	15 µL