RECOVERY PROCESS OF JACALIN FROM CRUDE PROTEIN OF JACKFRUIT SEED POWDER: OPTIMIZATION OF EXTRACTION AND CYTOTOXICITY STUDY OF JACALIN TOWARDS HUMAN CANCER CELL

ZURAIDAH BINTI MOHD ALI

DOCTOR OF PHILOSOPHY IN BIOPROCESS ENGINEERING

UNIVERSITI MALAYSIA PAHANG

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RECOVERY PROCESS OF JACALIN FROM CRUDE PROTEIN OF JACKFRUIT SEED POWDER: OPTIMIZATION OF EXTRACTION AND CYTOTOXICITY STUDY OF JACALIN TOWARDS HUMAN CANCER CELL

ZURAIDAH BINTI MOHD ALI

Thesis submitted in fulfilment of the requirements
for the award of the degree of
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Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

SEPTEMBER 2015

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Signature :
Name of Supervisor : PROF. DR. MIMI SAKINAH BINTI ABDUL MUNAIM
Position : PROFESSOR
Date : 15th SEPTEMBER 2015

Signature :
Name of Co-supervisor : ASST. PROF. DR. WAN AZIZI BIN WAN SULAIMAN
Position : SENIOR LECTURER
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In the name of Allah, The Most Gracious and The Most Merciful

I humbly dedicated this thesis to…

my dear husband; Mohd Rashiddin Abdul Rashid,
my lovely sons; Muhammad Azizul Hakim and Muhammad Amirul Haziq,
my little sweetie; Nur Aleesya Humaira and Nur A'arifa Hisyma,
my special encouraging parents; Ummie, Mak and Abah,
my remarkable family members,
my best friends,
those who has influenced my life on the right path

thank you very much

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ABSTRACT

Jacalin is a major lectin present in jackfruit seeds, obtained by crude protein recovery. Its lectin symbol is AIL and it belongs to the galactose family of N-acetylgalactosamine binding lectins, which are recognized to be cancer cell inhibitor. There have been many pharmacological research studies focusing intensively on jacalin, however their scope was restricted to the application of jacalin in pharmacology. Although there are methods for producing jacalin, they are still limited owing to its low productivity. Low yield production compared to high demands in the pharmacy industry increases the price of jacalin. An understanding of the recovery and extraction process as well as separation process behavior can be obtained via research and experimentation in order to determine the optimum extraction and separation conditions of jacalin production. The optimum conditions of crude protein recovery are very important as they contribute to attaining high yield extraction of jacalin as well. However, the optimum soaking pretreatment time was determined prior recovery process conducted. The parameters that mainly influence crude protein recovery are liquid to solid ratio (LSR), temperature and recovery time. The independent variables were investigated using One-Factorial-At-Time (OFAT) approach prior to optimizing the recovery process by response surface methodology (RSM). The recovery of crude protein with optimum conditions was followed by extraction of jacalin from crude protein. Liquid-liquid extraction by reversed micelles method was used in the extraction of jacalin from crude protein, by studying two variables; salt concentration (NaCl) and pH value. Crude protein and jacalin extract were applied to two cancer cell lines, human breast cancer cell (MCF7) and non-small lung carcinoma cell (H1299), in comparison to jacalin standard. In studying the soaking pretreatment time effect, it was determined that 6 hr was the optimum soaking time. Meanwhile, the OFAT study showed that three variables, LSR, temperature and recovery time were influence in crude protein recovery and the optimization was achieved using RSM, which 29.11 % increased with 42.84 mg/mL. The reversed micelles system was successfully used to extracting jacalin from the crude protein of jackfruit seed powder, with optimum pH of 6.0 and salt concentration up to 0.2 M resulting in jacalin concentrations of 1.782 mg/mL. The jacalin extract was more effective compared to crude protein in inhibiting the viability for both of cancer cell. IC_{50} for MCF7 was achieved at concentration 125 µg/mL, closely comparable to jacalin standard with only 1.60 % contradictions. Thus, reversed micelles extraction method can be proposed to use as a purification method for jacalin, besides, this method is easy to scale up and operate continuously. Furthermore, recommended that further research on others parameter effect of crude protein recovery such as particle size of jackfruit seed powder and using variety species of jackfruit seed.

ABSTRAK

Jakalin adalah lektin utama yang terkandung di dalam biji nangka diperolehi melalui proses pemerolehan semula protein mentah. Simbol lektinnya adalah AIL dalam kumpulan berikatan dengan N-asetilgalaktosamina dari keluarga galaktosa, ia sangat sesuai sebagai perencat sel barah. Banyak penyelidikan memfokuskan kepada aplikasi jakalin di dalam bidang farmasi tetapi kadar pengeluaran yang rendah dengan permintaan yang tinggi menyebabkan harga jakalin menjadi mahal. Memahami proses pemerolehan semula, pengekstrakkan dan pemisahan melalui penyelidikan dan ujikaji dapat mengekstrak jakalin dengan optima. Pemerolehan semula protein mentah yang optima sangat penting kerana ia mempengaruhi pengekstrakan jakalin yang tinggi. Namun, tempoh pra-rawatan rendaman yang optima juga dikenalpasti mempengaruhi proses pemerolehan semula protein mentah. Selain itu, parameter yang mempengaruhi hasil pemerolehan semula protein mentah adalah nisbah cecair kepada pepejal, suhu dan masa pemerolehan. Kebergantungan di antara pembolehubah dijalankan menggunakan pendekatan satu-faktor-satu-masa dan pengoptimaan pemerolehan semula protein mentah dijalankan menggunakan kaedah tindakbalas permukaan. Proses pengekstrakkan jacalin dari protein mentah menggunakan kaedah pengekstrakkan cecair-cecair oleh misel balikan dengan mengkaji dua pembolehubah iaitu kepekatan garam (NaCl) and nilai pH. Kemudian, protein mentah dan ekstrak jakalin diaplikasikan terhadap dua jenis sel iaitu sel barah payudara (MCF7) dan sel barah paru-paru (H1299) dan di bandingkan dengan jakalin piawai. Dalam mengkaji kesan tempoh pra-rawatan rendaman, didapati 6 jam adalah tempoh rendaman yang optima. Manakala, kajian satu-faktor-satu-masa telah menunjukkan bahawa tiga pembolehubah iaitu nisbah cecair kepada pepejal, suhu dan masa telah mempengaruhi hasil pemerolehan protein mentah dan pengoptimaan telah dicapai menggunakan kaedah tindakbalas permukaan dengan menghasilkan 42.84 mg/mL kepekatan protein mentah iaitu 29.11% melebihi nilai sebelum pengoptimaan. Sistem misel balikan berjaya digunakan untuk mengekstrak jakalin daripada protein mentah dengan memperolehi kepekatan jakalin optima sebanyak 1.782 mg/mL pada nilai pH 6 dan kepekatan garam 0.2M mg/mL. Ekstrak jakalin lebih efektif berbanding protein mentah dalam perencatan kedua-dua sel barah dan telah mencapai IC_{50} pada kepekatan 125 µg/mL, di mana ia hampir sama dengan kesan yang ditunjukkan oleh jakalin piawai dengan perbezaan hanya 1.60 %. Oleh itu, pengekstrakkan menggunakan misel balikkan dicadangkan digunakan sebagai kaedah penulenan jakalin. Selain itu, kaedah ini juga mudah dijalankan pada skala besar dan beroperasi secara berterusan. Kajian akan datang disyor mengkaji lebih banyak pembolehubah dalam proses pemerolehan semula seperti saiz serbuk biji nangka dan menggunakan biji nangka dari pelbagai spesis.

TABLE OF CONTENTS

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AOT	Sodium di(2-ethylhexyl) sulfosuccinate
BBD	Box-Behnken statistical experiment design
BSA	Bovine serum albumin
DEAE	Diethylaminoethanol
DNA	Deoxyribonucleic acid
DOE	Design of expert
FFD	Full factorial design
FTIR	Fourier transforms infrared spectrometer
HFS	Human skin fibroblast carcinoma
HIV	Human immunodeficiency virus
HPAEC	High performance anion-exchange chromatography
HPLC	high performance liquid chromatography
IEF	Isoelectric focusing
IgA	Immunoglobulin A
JSP	Jackfruit seed powder
LSR	Liquid to solid ratio
MALDI	Matrix assisted laser desorption/ionization
MAP30	Natural component from *Momordica charantia*
MARDI	Malaysia Agriculture and Research Development Institution
MCF7	Human breast cancer cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW	Molecular weight
OFAT	One-factor- at-time
PBS	Phosphate buffer saline
PNA	Peanut agglutinin
RME	Reverse micelle extraction
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
T-antigen	Thomsen-friendenreich antigen
TOF MS	Time of flight mass spectrometry
UV/Vis	*Ultraviolet–visible* spectroscopy or *ultraviolet-visible* spectrophotometr

LIST OF SYMBOLS

*	equilibrium
C_{aq}	Concentration of aqueous
C_{org}	Concentration organic phase
k_{aq}	Mass transfer coefficient in aqueous phase
k_b	Mass transfer coefficient for backward extraction
k_f	Mass transfer coefficient for forward extraction
k_{org}	Mass transfer coefficient in organic phase
'K'	Overall mass transfer coefficient
aq	Aqueous phase
IC_{50}	50% inhibition of cell viability
0	Initial concentration
org	Organic phase
A	Interfacial area
H	Partition coefficient
V	Volume of each phase

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Jacalin is a major lectin present in jackfruit seeds, usually obtained by crude protein extraction. Structurally, it consists of a lectin subunit combining a heavy chain (α) and a light chain (β), each containing 133 and 20 amino acid residues, respectively (Kabir, 1995). Jacalin has the lectin symbol AIL, belongs to the galactose family of N-acetylgalactosamine binding lectins, originates from *Artocarpus heterophyllus* (jackfruit) and has the ligand motif (Sia)Galβ1-3GalNAcα1-Ser/Thr. Galactose binding lectins are recognized to be highly specific for the Thomsen-Friendenreich antigen, generally known as T-antigen, which is expressed in more than 85 % of human carcinomas. Besides that, jacalin has received considerable attention on account of its interesting biological properties; it has been used extensively for the isolation of IgA from human serum and human plasma glycoproteins, application in pharmacology and is a useful tool for the evaluation of the immune status of patients infected with human immunodeficiency virus (HIV) (Kabir, 1998). In the production of a jacalin, crude protein is recovered from jackfruit seed powder (JSP) by phosphate buffer saline (PBS) precipitation and filtration. The crude protein contains total of protein, thus the separation process is necessary in order to extract the lectin known as jacalin from crude protein.

There are many pharmacological research studies focusing intensively on jacalin now days, however their scope is restricted to the application of jacalin in pharmacology. Although there are methods to extracting jacalin, these methods are still limited due to low production of jacalin (as function of operating time). Therefore reducing the production rate and increasing the cost of operation. Low yield production compared to high demands in the pharmacy industry increases the price of jacalin. An understanding of the recovery and extraction process as well as separation process behavior could be obtained to determine the optimum conditions of jacalin production.

1.2 PROBLEM STATEMENT

Thomsen-Friendenreich antigen, generally known as T-antigen, is generally expressed in more than 85 % of human carcinomas such as cancers of the colon, breast, bladder, buccal cavity and prostate. Therefore, only proteins that specifically bind T-antigens have potential diagnostic value for these cancers. On the market 2006, jacalin is sold in the form of salt free lyophilized powder for about USD 50 for 10 mg (Othman et al., 2006) and it was increasing till USD 80 on 2015 (www.medicago.se). Due to its good price and vast benefits, it is commercially attractive to recover jacalin from jackfruit seeds. Since jackfruit is abundant in Malaysia the whole year round, the seeds can be obtained easily. However, there are many problems to overcome during the preparation of jacalin, such as a low extraction rate, high extraction cost, difficulties in hand-run extraction and intensification, all of universally exist in the process of separation and purification of biomolecules which restrict the production of jacalin. Prior to the purification process, crude proteins are recovered from JSP using conventional methods that employ PBS precipitation and filtration. Nevertheless, no study has been carried out on the optimization of this process by using experimental design response surface methodology (RSM). The optimum condition of crude protein recovery is very important as it contributes to the high production of jacalin as well. The parameters that mainly influence crude protein recovery are liquid to solid ratio (LSR), temperature and recovery time. Inappropriate selection of recovery parameters would cause low yield of crude protein, therefore contributing to the low production of jacalin in the extraction process.

The method and type of purification process used also influence jacalin production. Chromatography methods that have been used to purify jacalin are anion exchange high performance liquid chromatography (HPLC) and affinity chromatography (Kabir, 1995). Purification from the crude protein by affinity chromatography is conducted on immobilized immunoglobulin A (IgA) as the ligand or on cross-linked guar gum, with both being eluted with galactose. Purification based on charge properties are subjected to anion-exchange chromatography on a diethylaminoethanol (DEAE)-matrix, and is carried out by high performance anion-exchange chromatography of the crude seed extract on a column of DEAE-polyacrylate. These methods involve high costs in order to prepare the suitable column and materials to be scaled up for commercial industries. Therefore, to solve these problems, alternative techniques have received particular attention. In this study, liquid-liquid extraction by reversed micelles was used as alternative methods to replace the purification of jacalin. Reversed micelles were used to extract jacalin from a crude protein, and the factors affecting reverse micellar systems, which are pH value and ionic strength or salt concentration, were studied. This process can be carried out without any specific equipment (e.g columns) and it is possible to be scaled up for commercial industries.

1.3 OBJECTIVES OF RESEARCH

The objectives of this research are :-

i) to determine the optimum recovery condition of crude protein from jackfruit seed powder for parameter liquid to solid ratio, temperature and recovery time using response surface methodology,

ii) to study a liquid-liquid extraction by reversed micelle as alternative method in the purification of crude protein to obtain a jacalin

iii) to examine application of a jacalin cytotoxicity towards human cancer cells.

1.4 SCOPES OF RESEARCH

The following are the scopes in this research that were identified and carried out to achieve the objectives:

i. Jackfruit seeds were separated from jackfruit flesh, clean, cut, sliced, dried and ground into powder. The main structural components of JSP were determined by standard methods.

ii. The JSP was characterized based on its functional groups by FTIR, microcosmic analysis by SEM and proximate composition by MARDI. The characterization of crude protein and jacalin extracted was analyzed based on absorbance concentration by UV-Vis, molecular weight by SDS-PAGE, functional groups by FTIR, protein sequences by MALDI TOF and cytotoxicity to cancer cell line by MTT assay.

iii. Prior to the recovery process, soaking pretreatment was applied in order to shorten recovery time. The highest concentration of crude protein recovered was chosen as the optimum pretreatment time.

iv. Crude protein was recovered from JSP using conventional methods that employed phosphate buffer saline, precipitation and filtration. The effective range of process factors, that is LSR, temperature and recovery time, was selected through the one-factor-at-time (OFAT) method using Design of Expert (DOE) software and the conditions were optimized with Response Surface Methodology (RSM).

v. Extraction of jacalin from crude protein was carried out using liquid-liquid extraction by reverse micelle method. A variety of processes using different pH and salt concentrations were performed to study the effects of both parameters on the extraction of jacalin from crude protein.

vi. Cytotoxicity effect of jacalin towards cancer cells was examined through determination of viability cells. The absorbance of this coloured solution was measured by spectrophotometer to calculate the living cells.

1.5 SIGNIFICANTS OF RESEARCH

Jacalin is growing in demand and is well known as one of the most recent and special lectins in the pharmaceutical and medical industries. Previous studies only focus on the application and function of jacalin but are inattentive on the optimization of recovery and purification processes. As a result, jacalin production currently faces a crucial problem in fulfilling pharmaceutical and medical industry demands. The conventional extraction methods have not yet been optimized and purification using chromatography is very costly and not practical for scaling up. The approach used in this study can overcome the drawbacks of the previously mentioned extraction and purification methods, which are exclusively used at present. This study was carried out to recover the crude protein from JSP and extraction of jacalin from the crude protein. The details of the significance of the present work are stated below:

i. Jackfruit seed is available at a reasonable cost through the year in Malaysia, because it was non-seasonal fruit. Jackfruit seed was a waste in the snack and canned food of jackfruit. Extraction of jacalin from jackfruit seeds could is a great alternative to manage this abundant waste. Furthermore, the utilization of jackfruit seeds as raw materials to extract jacalin has dual consequence: the utilization of waste and the generation of a high value product that would be helpful for sustainable environmental management and economic development.
ii. Optimization of the recovery process of crude protein and applied alternative method (reverse micelle extraction method), which is cheaper and easy to scale up in extraction of jacalin from crude protein will improve the production jacalin in market.
iii. Examining the application of jacalin to human cancer cells to determine new ways that cancer cell types can be inhibited and expanding the application of jacalin as well.

CHAPTER 2

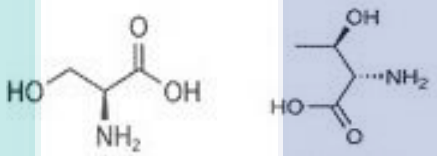
LITERATURE REVIEW

2.1 INTRODUCTION

This chapter is a review of previous studies that are relevant to jacalin production. The structure, sources, properties and application of jacalin are explained at the beginning of the chapter, followed by discussion of the main steps or processes in the extraction of jacalin starting with crude protein recovery from jackfruit seed powder (JSP), described in detail. This chapter also reviews optimization strategies for recovery steps, and the different methods of extracting crude protein as well as extracting jacalin are conversed, highlighting the advantages and disadvantages associated with each process scaled up for industrial application. In addition, this chapter compares between different jacalin extraction methods in order to determine the optimum jacalin yield. In particular, the main steps in the extraction of jacalin are recovery of crude protein from JSP by phosphate buffer saline (PBS) precipitation, followed by extraction of jacalin from crude protein. Furthermore, the pharmaceutical applications of jacalin, specifically in cancer cells, are reviewed and discussed.

2.2 JACALIN

Jacalin is a plant lectin from jackfruit or *A. heterophyllus* seeds, is a tetrameric two-chain lectin with a molecule structure weight of 12-17 kDa (Arslan and Chulavatnatol, 2000 and Pratap et. al., 2002) . It combines a heavy α chain of 133 amino acid residues with light β chain of 20 to 21 amino acid residues (Kabir, 1998; Jeyaprakash, 2002 and Swami et al., 2012). Jacalin is a galactose binding lectin type with symbol AIL and ligand motif (Sia)Galβ1-3GalNAcα1-Ser/Thr. Ser/Thr means of combination of amino acid of serine and threonine, which image showed in Figure 2.1. Table 2.1 list of major lectins with their symbols, names, sources and ligand motifs, respectively (Unitt and Hornigold, 2011; Delatorre et al., 2013 and Varrot et. al., 2013).



(a) (b)

Figure 2.1: (a) Serine (Ser) (b) Threonine (Thr)

Source: Houle (2002)

Table 2.1: List of major lectins with their symbols, names, sources and ligand motifs

Lectin symbol	Lectin Name	Sources	Ligand Motif
Mannose binding lectins			
ConA	Concanavalin A	*Canavalia ensiformis*	α-D-mannosyl and α-D-glucosyl residues Branched α-mannosidic structures (high α-mannose type or hybrid type and biantennary complex type N-glycans)
LCH	Lentil lectin	*Lens culinaris*	Fucosylated core region bi- and triantennary complex type N-glycans
GNA	Snowdrop lectin	*Galanthus nivalin*	A 1-3 and α 1-6 linked high mannose structures
Galactose / N-acetylgalactosamine binding lectins			
RCA	Ricin, Ricinus, Communis Agglutinin	*Ricinus Communis*	Galβ1-4GalNAcβ1-R
PNA	Peanut agglutinin	*Arachis hypogaea*	Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
AIL	Jacalin	*Artocarpus heterophyllus*	(Sia)Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
VVL	Hairy vetch lectin	*Vicia villosa*	GalNAcα-Ser/Thr (Tn-Antigen)
N-acetylglucosamine binding lectins			
WGA	Wheat Germ Agglutinin	*Triticum vulgaris*	GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)
N-acetylneuraminic acid binding lectins			
SNA	Elderberry lectin	*Sambucus nigra*	Neu5Acα2-6Gal(NAc)-R
MAL	*Maackia amurensis* leukoagglutinin	*Maackia amurensis*	Neu5Ac/Gcα2,3Galβ1,4Glc(NAc)
MAH	*Maackia amurensis* hemagglutinin	*Maackia amurensis*	Neu5Ac/Gcα2,3Galβ1,3(Neu5Acα2,6)GalNac
Fucose binding lectins			
UEA	*Ulex europaeus* agglutinin	*Ulex europaeus*	Fucα1-2Gal-R
AAL	*Aleuria aurantia* lectin	*Aleuria aurantia*	Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1-4GlcNAc,R2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R1

Sources : Unitt and Hornigold, (2011); Delatorre et al., (2013) and Varrot et. al., (2013)

2.2.1 Natural Source of Jacalin

Jacalins are the single most major lectin identified in the jackfruit seed, and towards the end of the 1970s more than 50 % of lectins were extracted from jackfruit seed powder (Kabir, 1998). Seeds were processed (collected from fresh jackfruit, cleaned, sliced, dried and grinded) into powder before jacalin extraction (Ajayi, 2008). In Malaysia, jackfruit is a non-seasonal fruit and the seeds are easy to obtain at a low price since they are considered to be waste products in the food industry.

2.2.2 Applications of Jacalin

Jacalin, a unique lectin present in jackfruit seeds, has a wide range of activities (Jagtap and Bapat, 2010). Jacalin has been reported to bind specifically to human IgA, D-galactose and Thomsen-Friedenreich antigen (T-antigen). It is highly specific for the O-glycoside of the disaccharide Thomsen-Friendenreich (Galβ1-3GalNAc). This property has made jacalin is glycosylated thus it has potential value in studying various O-linked glycoproteins, particularly human IgA_1 including isolation of human plasma glycoproteins, investigation of IgA nephropathy and detection of tumors (Swami et al., 2012). Subsequent studies have revealed that jackfruit seed extract possesses a potent and selective mitogenic effect on T-cell proliferation and B-cell polyclonal activation (Kabir, 1998 and Jagtap and Bapat, 2010). Traditionally, extract from fresh seeds cures diarrhea, dysentery and helps digestion (Swami et al., 2012).

Interdisciplinary programmes that incorporate conventional and new technologies will be critical for the future development of jackfruit seed as a promising source of medicinal products. As covered in this review, important findings have been made in regards to identification; synthesis and bioactivity of the metabolites present in jacalin, which are highlighted in this review along with the current trends in research. Table 2.2 shows the applications of jacalin from jackfruit seed in phytochemical and biochemical studies.

Table 2.2: Phytochemical and biochemical studies on jacalin

References	Focus of the phytochemical and biochemical studies
(Ahmed and Chatterjee, 1989)	Jacalin was demonstrated to stimulate both human T and B lymphocytes and precipitated among five classes of Igs only IgA and IgD.
(Kabir, 1995)	Highly specific for the tumour- associated Thomsen-Friedenreich antigen, having Gal beta(1-3) GalNAc structure, considerable attention of immunologists as it binds immunoglobulin A (IgA), subclass specificity being restricted to human IgAl. Jacalin has found to have diverse biomedical applications such as isolation of IgAl from serum investigations of O-linked oligosaccharides purification of C-l inhibitor and detection of breast and thyroid tumours Block HIV-l in vitro infection of lymphoid cells, thus becoming a tool in AIDS research.
(Kabir, 1998)	In recent years jacalin has been applied in AIDS research since it is the only lectin known to be selectively mitogenic for human CD4+ T cells. Since CD4+ T lymphocytes act as the receptor of HIV-1, jacalin has been used to investigate the proliferation of peripheral blood mononuclear cells (PBMC) and in HIV-1 infected patients.
(Jeyaprakash, 2002)	Jacalin binds to the sialylated derivatives of T-antigen and Tn-antigen (GalNAc-Ser/Thr), which are termed cryptic T- and Tn-antigens, respectively. Selectively binds to the a-linked forms of T-antigen and mitogenic for human CD4+ T-cells, used in AIDS study. Extensively used for the isolation of IgA and other glycoproteins including carcinoma related mucins
(Jeyaprakash et al., 2003)	Jacalin shows interesting biological properties, it selectively binds IgA1 and other glycoproteins such as carcinoma-related mucins. Selectively mitogenic for human CD4+ T-cells, therefore useful in AIDS research. As a tool to investigate IgA1 nephropathy, to identify O-linked glycoproteins and in histochemistry.
(Tachibana et al., 2006)	Valuable tool for specific capturing of O-glycoproteins such as mucins and IgA1. Though its sugar-binding preference for T/Tn-antigens is well established, its detailed specificity has not been elucidated
(Pandey et al., 2009)	Jacalin binds phycocyanin (PC), specifically in a carbohydrate-independent manner and with affinities better than that for porphyrins. Hence, the lectin could prove to be a useful carrier for targeted delivery of PC.
(Uraya et al., 2011)	Jacalin is a lectin that recognizes sugars on IgA1.

2.2.3 Jacalin as Lectin

Lectins are proteins structurally diverse carbohydrate-binding proteins of polysaccharides, glycoproteins or glycolipids from non-immune origin that are able to agglutinate cells or precipitate carbohydrates, without having enzymatic activity towards their carbohydrate. They contain two or more binding sites and can agglutinate cells and/or precipitate complex carbohydrate conjugates. They have been isolated from various plant, animal and bacteria sources, and are widely used in biochemical and cellular studies (Arslan and Chulavatnatol, 2000 and daSilva et al., 2005). Plants are the richest and most convenient source of lectins, and plant-sourced lectins have been attracting much attention because of their ease of isolation and usefulness as reagents for glycoconjugates in solutions and on the cell surface (Laija et al., 2010).

Lectins have been revealed to have varying degrees of specificity based on sugar content, conformation and bonding; such as concanavalin A recognizes α-D-mannose and α -D-glucose, whereas wheat germ agglutinin binds to sialic acid and molecules containing N-acetyl-D-glucosamine residue and jacalin from jackfruit seed binds to a-D-galactosyl groups, which are among the best characterized seed legume (Ayyar et al., 2012). Jacalins are glycoproteins containing approximately 7 to 10 % carbohydrate (Kabir, 1998).

In recent years, lectins have become very attractive due to their extensive use as probes for both the characterization and isolation of simple and complex sugars as well as useful tools in immunological studies. Considering the innumerable amount of lectins available in nature, the ease with which they can be prepared in purified form. Their flexibility to chemical manipulation and the fact that they can be inhibited by simple sugars makes them attractive as an important tool in biological research. Although lectins are found universally in plant species, they have variable structures and specific activities according to the plants they originate from. Thus, purification and characterization of lectins from a variety of plant species interests many researchers in the field of glycobiology.

2.3 JACKFRUIT

Jackfruit or scientific name *Artocarpus heterophyllus Lam*, belongs to the family *Moraceae* (mulberry family). It is popularly known as jackfruit or jak-fruit, jak, and nangka in Malaysia; jaca in the Philippines; khanun in Thailand; khnor in Cambodia; mak mi or may mi in Laos; and mit in Vietnam (Baliga et al., 2011). There are a few types or species of jackfruit called *nangka madu*, for example *nangka madu CJ1, CJ2, CJ3, CJ4, CJ5, CJ6* and *Mastura*. This study uses the jackfruit species *Mastura* which is easy to obtain, commercialized, highest in demand and is a non-seasonal species. According to Jabatan Pertanian Malaysia, planting of jackfruit in commercial amount was starting on 2008, with 100 hectare farms at Selama, Perak and in 2010, about 4000 hectares new farm at Temerloh, Kota Tinggi and Segamat. Approximately, it can produce about 12,000 tons per year of jackfruits.

The jackfruit tree, also known as the *Ceylon Jack* tree, is one of the most important and commonly found trees in the home gardens of India and Bangladesh (Baliga et al., 2011), as well as being popular in several tropical countries. The jackfruit tree is one of the most important trees in tropical home gardens and possibly the most widespread and useful tree in the genus *Artocarpus.* The jackfruit tree is a wild plant and is a medium-sized evergreen tree, typically reaching 8 to 25 m in height, and is easily recognized by its fruit. It is the largest known edible fruit up to 45 kg in weight, up to 90 cm in length and 50 cm in diameter. Each fruit is oblong cylindrical in shape with 30 to 40 cm in length. A mature tree can produce anywhere between 10 to 200 fruits at a time.

The jackfruit, which as a young fruit can be used as a vegetable, has several uses. The flesh of ripe fruit is used in various ways; it can be eaten raw as a salad with its juicy, aromatic, and tasty flavor or preserved in numerous ways for example inside cans as jam or dried fruit (Babitha et al., 2006; Elevitch and Manner, 2006 and Baliga et al., 2011). Various parts of the plant including the bark, roots, leaves, and fruit are attributed with medicinal properties. Meanwhile, the nutritious seeds are boiled or roasted and eaten like chestnuts (Elevitch and Manner, 2006).

Previous studies have shown that jackfruit contains many classes of compounds, for example carotenoids, flavonoids, volatile acid sterols and tannins, and the concentrations of these compounds alter with different types. Carotenoids are known to impart a yellowish-red color to many foods and their ratio is supposed to render the jackfruit various yellow to orange shades of color. The kernel is reported to contain β-carotene, α-carotene, β-zeacarotene, α-zeacarotene, β-carotene-5,6 α-epoxide, dicarboxylic carotenoid and crocetin (Baliga et al., 2011). The jackfruit is a rich source of phytochemicals, including phenolic compounds and useful antioxidant compounds, hence offering opportunities for the development of value-added products from jackfruit, as well as pharmaceutical and food applications to enhance health benefits (Jagtap et al., 2010). Ripe fruit pulp has a high nutrient value, a caloric value of 84 calories, and comprises of 18.90 % carbohydrate, 1.90 % protein, 0.10 % fat, 77.00 % moisture, 1.10 % fiber and 0.80 % total mineral matter, consisting of calcium, phosphorus, iron, vitamin A and thiamin (Swami et al., 2012).

2.3.1 Jackfruit Seed

The shape of the jackfruit seed is oval, rounded, oblong or oblong ellipsoid. The seeds are naturally light brown in colour, 2.00 to 3.00 cm in length and 1.00 to 1.50 cm in diameter (Figure 2.4). Jackfruit seeds are normally discarded or steamed and eaten as a snack or used in some local dishes (Babitha et al., 2006). Jackfruit seeds contribute around 10 to 15 % of the total fruit weight, with up to 500 pieces of seeds in each fruit. Seeds are separated by horny endocarpus enclosed by a sub-gelatinous exocarpus, a thin whitish membrane (1 mm thickness).

Seed flour is an alternative product which is used in some food products (Tulyathan et al., 2002). For this research, seed powder was prepared from fresh jackfruit seeds of the Madu Mastura jackfruit species. In the process of preparing seed powder, the seeds are separated from the fruit flesh, the latex is cleaned, then the seed is sliced and dried to become chips. The chips are grinded and crushed well and uniformly with maximum precaution to avoid any contamination, then the powdered materials are packed into plastic pouches and stored in normal room temperature until use (Babitha et

al., 2006). The seed is made out of a starch-based material that originally attracted a great deal of interest because of their low cost, real biodegradability, and renewable origins. The seed starch is given to relieve nausea. Roasted seeds are regarded as an aphrodisiac (Swami et al., 2012).

The seeds are boiled or roasted and eaten like chestnuts, added to flour for baking, or cooked in dishes. Jackfruit seed powder comprises manganese and magnesium elements. Besides, the seeds also contained lectins, namely jacalin. Jacalin has proven to be useful in phytochemical and biochemical studies, for example nanosized particles of jackfruit seeds were found to have an antibacterial effect against *E. coli* and *B.megaterium* microbes, revealing the efficacy of jackfruit seed nanoparticles as an antibacterial agent. Jackfruit seeds may therefore be developed into therapeutic agents capable of treating infectious diseases and preventing food contamination by food-borne pathogens. Jackfruit seeds could also be processed into dual-functional food ingredients possessing antimicrobial activities (Swami et al., 2012).

2.3.2 Composition of Jackfruit Seeds

Jackfruit seeds are nutritious, rich in potassium, fat, carbohydrates, minerals, protein, calcium and thiamine. Manganese and magnesium elements have also been detected in jackfruit seed powder. Ajayi (2008) mentioned that carbohydrates obtained from jackfruit seeds could act as source of energy for animals if included in their diets. The seed contain the oil which the oils are consistently liquid at room temperature. Studies have revealed potassium to be the dominant mineral element, followed by sodium, magnesium and then calcium. The seeds also contain a reasonable quantity of iron, high calcium, thiamine and protein, which is jacalin (Elevitch and Manner, 2006).

However, according to Baliga et al. (2011), composition in seed is different compared to fruit (flesh) of jackfruit. Tables 2.3 show the proximate composition of young fruit, ripe fruit and seed of jackfruit on the basis of 100 g fresh weight, respectively. Besides the composition of jackfruit seed is different depending on the location and types of jackfruit. The Table 2.4 showed comparison of nutrient composition of the jackfruit seed from three sources, Ajayi (2008), Devalaraja et al. (2011) and Baliga et al. (2011). Meanwhile Table 2.5 shows the proximate composition (% dry matter) of *Artocarpus heterophyllus*, *Treculia africana*, groundnut, palm kernel, pumpkin, *Vinorama A.contricta*, papaya and *Bauhinia froficata* seeds.

Table 2.3: Proximate composition of young fruit, ripe fruit and seed of jackfruit on 100 g fresh weight

Composition	Young Fruit	Ripe Fruit	Seed
Moisture (g)	76.2–85.2	72.0–94.0	51.0–64.5
Crude fat (g)	2.0–2.6	1.2–1.9	6.6–7.04
Crude protein (g)	0.1–0.6	0.1–0.4	0.40–0.43
Carbohydrate(g)	9.4–11.5	16.0–25.4	25.8–38.4
Fiber (g)	2.6–3.6	1.0–1.5	1.0–1.5
Minerals			
Total Minerals (mg)	0.9	0.87–0.9	0.9–1.2
Calcium (mg)	30.0–73.2	20.0–37.0	50
Magnesium (mg)	-	27	54
Phosphorus (mg)	20.0–57.2	38.0–41.0	38.0–97.0
Potassium (mg)	287–323	191–407	246
Sodium (mg)	3.0–35.0	2.0–41.0	63.2
Iron (mg)	0.4–1.9	0.5–1.1	1.5
Vitamins			
Vitamin A (IU)	30	175–540	Not mention
Thiamine (mg)	0.05–0.15	0.03–0.09	0.25
Riboflavin (mg)	0.05–0.20	0.05–0.40	0.11–0.30
Vitamin C (mg)	12.0–14.0	7.0–10.0	11.0
Energy (Kj)	50–210	88–410	133–139

Source: Baliga et al. (2011)

Table 2.4: Nutrient composition of jackfruit seed

Nutrient (Units)	100 g of jackfruit seed		
Proximate			
Moisture (g)	2.78	72.23	57.25
Ash (g)	6.72	1.00	Not mentioned
Crude Protein (g)	20.19	1.47	6.80
Crude oil/fat (g)	11.39	0.30	0.42
Crude fiber (g)	7.10	1.60	3.19
Carbohydrate (g)	51.82	24.01	32.10
Energy (kcal or kJ)	Not mentioned	94.00	136.00
Minerals			
Calcium, Ca (mg)	19.00	34.00	50.00
Sodium, Na (mg)	39.85	3.00	63.20
Magnesium, Mg (mg)	24.00	37.00	338.00
Potassium, K (mg)	247.00	303.00	246.00
Iron, Fe (mg)	14.85	0.60	13.07
Copper, Cu (mg)	2.20	0.19	Not mentioned
Zinc, Zn (mg)	4.09	0.42	Not mentioned
References	Ajayi, 2008	Devalaraja et al., 2011	Baliga et al., 2011

Table 2.5: Proximate composition (% dry matter) of *Artocarpus heterophyllus*, *Treculia africana*, groundnut, palm kernel, pumpkin, *Acacia constricta (Vinorama)*, papaya and *Bauhinia froficata* seeds.

Parameter	**Species**							
	Artocarpus heterophyllus	*Treculia africana*	Groundnut	Palm kernel	Pumpkin	*Acacia constricta (Vinorama)*	Papaya	*Bauhinia froficata*
Moisture	2.78	3.78	4.45	14.26	NA	NA	0.09	NA
Ash	6.78	5.56	2.75	1.50	NA	NA	8.8	NA
Crude Protein	20.19	27.44	26.50	6.94	7.84	4.90 mg/mL	24.3	2.273 mg/mL
Crude fat	11.39	18.54	40.83	54.18	NA	NA	25.3	NA
Crude fiber	7.10	8.20	-	-	NA	NA	17.0	NA
Carbohydrate	51.82	36.48	25.40	23.10	NA	NA	32.5	NA
References	(Ajayi, 2008)	(Ajayi, 2008)	(Ajayi, 2008)	(Ajayi, 2008)	(Quanhong and Caili, 2005)	(Guzman et al., 2004)	(Unuabonah et al., 2009)	(Silva et al., 2012)

2.4 PROTEIN RECOVERY

Protein recovery is a separation stages in which the liquid (crude protein) is separated from the solid (jackfruit seed powder). The step in the protein recovery involves distillation, extraction, absorption, crystallization, adsorption and membrane processes, all of which are used to obtain the product at the required purity (Letcher, 2004). The recovery of antioxidants from natural sources is desired, since these bioactive substances are often used in functional foods, food additives and in the pharmaceutical as well as cosmetic industries. Yield is dependent on the solvent and method of recovery which must enable complete extraction of the needed compound while at the same time minimizing undesired products (Farsi and Lee, 2008). A lot of effort has been spent finding the most suitable extraction method with the highest yield, while also taking into consideration the safety and quality of the product (Yazan et al., 2011). Table 2.6 shows a comparison between different protein recovery processes for seeds of jackfruit or *Artocarpus heterophyllus*, Tepary bean, *Salvia bogotensis*, *Mimosa invisa* and *Acacia constricta (Vinorama).*

Extraction of jacalin is dependent on the effectiveness of crude protein recovery from jackfruit seed. The higher crude protein recovered, the higher the chances of extracting jacalin. The following represents a typical conventional method of crude protein recovery from jackfruit seed powder that employs phosphate-buffered saline (PBS), precipitating and filtration of the liquid supernatant. According to the summary of previous studies as shown in Table 2.6, the recovery process involves a long period of stirring in order to optimize yield. Thus, recovery efficiency can be improved by applying soaking pretreatment prior to the process, thereby shortening the recovery (stirring) period.

Table 2.6: Comparison between protein recovery methods of several seeds and lectins

Seed (Lectin)	Protein Recovery Method	Result and Discussion	References
Mimosa invisa (MI Lectin)	• Extracted with potassium phosphate buffer, • LSR 10 (mL/g) • Temperature : Room temperature • Centrifuged: 27,000 rpm for1 hr at at 4 °C. • Filtered through cheesecloth	• Crude protein recovery was 8% of seed. • Dialysis of the crude protein resulted in precipitation of the protein.	(Chandrika and Shaila, 1987)
Tepary bean or *Phaseolus acutifolius* (Arcelin)	• Prechilled of sodium acetate buffer added to flour • LSR 10 (mL/g) • Centrifuged: 20,000 rpm, for 10 min at 4 ^{o}C • Dialyzed for 24-48 hr at 4 ^{o}C	• Crude protein recovery was 7% of seed.	(Shade et al., 1990)
Jackfruit or *Artocarpus Heterophyllus* (Jacalin)	Extracted with PBS (0.01 M, pH 7.4, 250 mL) LSR 10 (mL/g) Stirring for 24 hr at 4 °C. Centrifuged: 10,000 rpm for 20 min at 4 ^{o}C. Filtered through a 0.45 µm filter	• Crude protein recovery was 8% of seed.	(Kabir, 1995)
Acacia constricta (Vinorama lectin)	• Defatted with hexane • Suspended in a NaCl solution • LSR 10 (mL/g) • Stirred for 2 hr at 4°C • Centrifuged: 1,480 rpm for 20 min at 4 ^{o}C • Extract clarified by glass fiber filtration and kept at 4 °C until use.	• Crude protein recovery was 5% of seed.	(Guzman et al., 2004)

Table 2.6: Continued

Seed (Lectin)	Protein Recovery Method	Result and Discussion	References
Tubers of Voodoo lily *(Sauromatum venosum* lectin*)*	• Soaking in PBS overnight at 4 ^{o}C and homogenized in a Waring blender • LSR 5 (mL/g) • Centrifuged: 20,000 rpm for 20 min at 4 ^{o}C.	• Recovery of crude protein was 22 % from sample	(Bains et al., 2005)
Crataeva tapia bark	• Extracted in NaCl • LSR 15 (mL/g) • Agitation overnight at 4 ^{o}C. • Filtered through gauze • Centrifuged: 4,000 rpm for 15 min at 4^{o}C • The supernatant was crude extract termed	• Recovery of crude protein was 56 % with retention and 80 % of the lectin hemagglutinating activity	(Nascimento et al., 2008)
Mucuna sloanei seeds	• Not mention	• Recovery of crude protein was 21.20 % from sample	(Teixeira-Sá et al., 2009)
Pineapple or *Ananas comosus L. Merryl* (Bromelain)	• Extracted in sodium phosphate buffer • LSR 10 (mL/g) • Filtered using cheese cloth). • Centrifuged : 10,000 rpm for15 min	• Measuring absorbance at 280 nm using BSA. • Protein concentration readings were taken in triplicate	(Kumar et al., 2011)
Small black kidney beans or *Phaseolus vulgaris*	• Mixed with 10 mM PBS (pH 7.0, • LSR 10 (mL/g) • Agitation overnight at 4 ^{o}C • Centrifuged : 9,000 rpm for 60 min at 4 ^{o}C	• Recovery of crude protein was 53.28 % from sample	(He et la., 2013)

LSR = Liquid to Solid ratio

2.4.1 Soaking Pretreatment

Pretreatment is a prerequisite step prior to the main protein recovery process, with the objective of preparing the condition of the sample before proceeding to the next process. There are many different pretreatment techniques, including drastic physical, chemical and biological approaches, which have been investigated over the past few decades, but there is no report that systematically compares the performances of these pretreatment methods. The beneficial effects of pretreatment have been recognized for a long time, but the choice of pretreatment technologies must consider several factors. An effective pretreatment method is needed that will reduce obstacles or produce multiple desirable effects (Karki et al., 2011; Peerajit et al., 2012 and Zheng et al., 2014).

As mentioned by Ali et al., (2009), the effects of pretreatment on sorghum grains in alkali bring about readjustments in the protein fractions. Meanwhile, other pretreatment methods such as particle size reduction also gave different yields of dietary fiber powder from lime residue (Peerajit et al., 2012). A pretreatment process is necessary to disrupt the naturally intractable carbohydrate lignin shields that impair the accessibility of enzymes and microbes to cellulose and hemicellulose, as reported by Zheng et al. (2014). Soaking in distilled water was found to be more effective at removing phytic acid from pulses than in solution of sodium bicarbonate, however, soaking in acidic buffer was more effective at removing phytic acid from brown rice and rice bran than in demineralised water, presumably because of the higher solubility of phytates in acidic conditions (Liang et al., 2009). These soaking pretreatments significantly increase the softness of the sample and also reduce the time period of the main process, thus reduce the downstream processing cost. It also enhances the recovery of proteins by increasing diffusivity in the solvent through an increase in the liquid phase, or even in some instances breaking the cell walls prior to the recovery process. This allows the proteins trapped in the cell to be released easier since there is less resistance to mass transfer presence (Zuraidah, 2010). Table 2.7 showed the summaries of comparison of pretreatment process involved prior recovery process of crude protein from jackfruit seeds.

Table 2.7: Summaries of pretreatment process prior recovery process of crude protein

Solid to liquid ratio (g:mL)	Pretreatment	Recovery Process	Centrifuge	Yield of crude protein	References
1:10	No pretreatment	Stirred for 24 hr in PBS at 4 ^{o}C	10,000 rpm, 20 min, 4 ^{o}C	Not mention	(Kabir et al., 1993)
1:10	No pretreatment	Stirred for 24 hr in PBS at 4 ^{o}C	10,000 rpm, 20 min, 4 ^{o}C	8mg/mL	(Kabir, 1995)
1:10	Overnight soaking in distilled water at 2^{o}C	Homogenized in a Waring blender in 0.1 M Tris/HCl	9,000 rpm, 15 min,	Not mention	(Houle, 2002)
1:6	24 hr soaking for in PBS	Homogenized with PBS 10 min in a Waring blender and stirred for 3 hr	8,000 rpm, 20 min, 4^{o}C	88 mg/mL	(Roy et al., 2005)
1:4	No pretreatment	Stirred in NaCl and left 30 min	10,000 rpm, 20min, 7 ^{o}C	0.47 mg/mL	(Othman et. al., 2006)
1:10	24 hr soaking in petroleum ether at room temperature	Stirred well with PBS overnight at 4 °C	10,000 rpm, 30 min	Not mention	(Laija et al., 2010)
Not mention	No pretreatment	48 hr filtrated deionized water in a cellulose acetate tube at 4 °C	No centrifuge	Not mention	(Uraya et al., 2011)
1:10	No pretreatment	Boiling in sterile water for 1 hr.	10,000 rpm for 15 min	Not mention	(Jagtap and Bapat, 2013)
Not mention	No pretreatment	Stirred for 12 hr at 4 ^{o}C	4,000 rpm.	Not mention	(Marangoni et al., 2013)

2.4.2 Phosphate Buffer Saline

Phosphate buffer saline (PBS) is a water-based salt buffer solution containing sodium phosphate and in some formulations, potassium chloride and potassium phosphate. It is a non-toxic, salty solution that prevents cells from rupturing or shrinking up due to osmosis to ensuring that the protein structure is preserved so that tissues, cells, and remain undamaged during the softening process. The uses for this solution include substance dilution, cell container rinsing and the removal of unbound proteins (George et al., 2011).

PBS can be stored at room temperature, but may require refrigeration to prevent bacterial growth if the solution is not sterile and is kept for long periods of time. Sterilization may not be necessary depending on its use. However, concentrated stock solutions may precipitate when cooled and should be kept at room temperature until the precipitate has completely dissolved before use. Occasional shaking brings about rapid equilibrium between intra- and extracellular fluids, thereby providing fresh protein for further purification. Sufficient time must be allowed for the protein to diffuse through the cell wall to solubilize the constituents present within the cells and for the resulting solution to diffuse out. The mixed substances go through the centrifugation process to separate supernatant and sediment residue, then they are stood for a certain amount of time to allow sediments to form precipitates to be clarified by filtration. There are many different ways of preparing a PBS solution and the simplest way to is to use PBS buffer tablets. They are formulated to give a ready to use PBS solution upon dissolution in a specified quantity of distilled water. They are available in the standard volumes: 100, 200, 500 and 1000 mL. Ready-made analytical grade PBS solutions for analytical use such as cell culturing are commercially sold. However, for this study PBS was manually prepared as will be explained in detail in Chapter 3.

2.5 JACALIN EXTRACTION METHOD

Extraction of a single protein from cells containing a mixture of thousands of unrelated proteins is achievable because of the remarkable variation in the physical and chemical attributes of proteins. Characteristics unique to each protein, such as amino acid composition, sequence, subunit structure, size, shape, net charge, isoelectric point, solubility, heat-stability, hydrophobicity, ligand/metal binding properties and post-translational modifications, can be exploited in formulation of a strategy for purification. Extraction enables the separation of selected compounds from dilute solutions and avoids using toxic solvents to minimizes degradation (Soto et. al., 2011).

Lectins have been extracted by ‘conventional’ methods including salt-induced crystallization, ethanol precipitation, dialysis using cellulose tubing, ion exchange chromatography, gel filtration by affinity chromatography and reverse micelle (Hou et

al., 2010; Jinjun et al., 2010 and Kabir, 1995). The previously mentioned methods rely on the physicochemical properties of the proteins for separation, while affinity chromatography depends on the specific interactions between the lectin and a carbohydrate structure attached to an inert matrix. Beside affinity chromatography, the most popular method for the purification of proteins and other charged molecules is ion exchange chromatography. Nevertheless, the reverse micelles extraction (RME) method is also utilized for extraction and purification as an alternative to chromatographic techniques, since the system can be easily scaled up and also allows for continuous separation processes, similar to the liquid-liquid extraction processes, which are commonly used in the chemical industry. Table 2.8 shows a comparison between multiple extraction methods of jacalin, and the next section will further explains each method of extraction in detail.

Table 2.8: Comparison lectin extraction method for several types of seed

Seed (Lectin)	Extraction Methods	Conclusion	References
Mimosa Invisa L (MI lectin).	• Purify by gel filtration and preparative Polyacrylamide gel electrophoresis • Affinity chromatography using column ConA-Sepharose • Elution of the bound lectin was tried with 0·1 M glucose, 0·1 M α-methyl-D- glucoside in 1 M NaCl, 0·1 M α-methyl-D-mannoside + 50 % (v/v) ethylene glycol, acetic acid-sodium acetate buffer, pH 3·6 with 1 M NaCl and 1 mM each of CaCl2, $MnCl_2$ and $MgCl_2$, and detergent buffer (1·4 % cetyltrimethyl ammonium bromide 1 M NaCl in 50 mM acetic acid-sodium acetate buffer).	• Crude extract was 0.8 mg/mL • Total activity was 160 units • Specific activity was 2 units/mg protein • Non-specifically agglutinates human erythrocytes. • Bound irreversibly to ConA-Sepharose and displacement of the bound protein was difficult. • Hydrophobic interactions may be responsible for the poor elution with α-methyl-D-glucoside. • The lectin showed affinity towards Sephadex G-100 and G-200	(Chandrika and Shaila, 1987)
Tepary bean or *Phaseolus acutifolius* (Arcelin)	• Affinity chromatography • Column ConA- Sepharose • HPLC Ion-Exchange Chromatography (Protein fractions were eluted with a 0 to 1.0 M NaCl gradient, 40 min, rate of 3 mL/min, monitored at 280 nm and recorded on an HP)	• Polypeptides from seed were 70 % enriched • Some proteins did not bind to the column or eluted during purification by chromatography • Purification of the polypeptides was attempted with thyroglobulin-agarose affinity chromatography.	(Shade et al., 1990)
Jackfruit or *Artocarpus Heterophyllus* (Jacalin)	• Affinity chromatography • IgA-Sepharose 4B column prepared by coupling human IgA (225 mg) to CNBr-activated Sepharose 4B (40 mL, Pharmacia, Uppsala). • Ion–exchange chromatography on DEAE-cellulose. The PBS extracts of jackfruit seeds (8 mL) were extensively dialysed against phosphate buffer (PBS, 0.01 M, pH 7.4) and were applied onto a column containing DEAE-cellulose (2.5 x 50 cm). • The adsorbed protein was eluted by PBS (0.01 M, pH 7.4) containing a stepwise gradient of 50, 100, 150, 200 and 500 mM NaCl.	• Protein content 8 mg/mL • Crude protein further purified by passage over a column containing human IgA coupled to Sepharose 4B. The bound materials were desorbed from the column with 0.5 M D-galactose. • Average yield of 30 % of jacalin of crude protein • Purification followed by isoelectric focusing by on ultrathin layer polyacrylamide gels using the pH gradient 3.5-9.5 • Jacalin also demonstrated multiple bands in the pH range of 5.0-8.5.	(Kabir, 1995)

Table 2.8: Continued

Seed (Lectin)	Extraction Methods	Conclusion	References
Acacia constricta (Vinorama lectin)	• Purification using affinity chromatography on a fetuin-fractogel column followed by cationic-exchange chromatography. • 2 mL of the resin added to 46 mg of fetuin previously suspended in 8 mL of phosphate or citrate buffer • The reaction mixture was incubated overnight at room temperature under constant agitation • Gel exclusion chromatography using a column of Superdex 200.	• Crude extract had a protein 4.9 mg/mL and specific activity of 419 units/mg per mL protein. • This fraction represented at least 4.2 % of the total extractable seed protein, which is similar to other non- cultivated desert legumes • Purify isolectins, lectin from *A. constricta* using affinity and cation-exchange chromatography	(Guzman et al., 2004)
Camaratu bean or *Cratylia mollis*	• Liquid-liquid extraction by reverse micelle • Anionic surfactant used AOT in isooctane. • Forward and back- extraction assays were performed by phase contact (1:1), stirred (5 min) and separated by centrifugation (3000 rpm,10 min)	• *C. mollis* seed lectin is possible to extract using reverse micelles of AOT (100 mM) in isooctane. • The pH is a major determinant parameter in the partitioning of lectin in the biphasic system.	(Nascimento et al., 2002)
Pineapple or *Ananas comosus L. Merryl* (Bromelain) .	• Liquid-liquid extraction by reverse micelle • Forward extraction was carried out by mixing equal volumes of organic phase (CTAB/80 % (v/v) isooctane/5 % (v/v) hexanol/15 % (v/v) butanol) with aqueous phase for 1 hr on magnetic stirrer. • Back extraction was carried out by mixing of reverse micelle phase with fresh stripping solution. • For the back extraction, pH of the aqueous phase was adjusted and mixed with fresh organic phase (AOT/isooctane) by magnetic stirring for 30 min. • Experiments were carried out at 25 ± 2 ^{o}C.	• Affinity based reverse micelle extraction separation could be successfully applied for the selective extraction and purification of bromelain from pineapple waste. • The optimized conditions for extraction resulted an activity recovery of 185.6 %. The forward, back, reverse and overall extraction efficiencies within 49 to 14 % were obtained.	(Kumar et al., 2011)

Table 2.8: Continued

Seed (Lectin)	Extraction Methods	Conclusion	References
Crataeva tapia bark	• Liquid-liquid extraction by reverse micelles. • Anionic surfactant used AOT in isooctane. • Stirred for 5 min for protein extraction and centrifuged for 5 min at 3000 rpm, for phase separation. • Back extraction, added to an equivalent volume of KCl at different pH containing and 5 % of butanol was added to the system. • Mixture stirred for 5 min, centrifuged for 5 min at 3000 rpm for phase separation and the lectin recovered in the new aqueous phase.	• AOT in isooctane system with the addition of butanol on the back-extraction step and adjustment of pH, ionic strength and AOT concentration. • Maximum recovery, 85 % was found at pH 7.0 with 500 mM KCl, • Agitation 700 rpm, temperature 25 ^{o}C and protein concentration 0.374 mg/mL	(Nascimento et al., 2008)
Red kidney beans	• Liquid-liquid extraction by reverse micelle • Anionic surfactant used AOT in isooctane. • Extraction and back extraction assays were performed by phase contact 1:1 (v/v), stirred for 5 min. • Separation by centrifugation at 2,292 rpm, 5 min.	• The pH value of the aqueous phase affected both forward and back extraction with the optimal pHs were 4–6 and 9–11, respectively. • Extraction time also affected reverse micelle extraction. The highest lectin was at 4–6 min. • The ion strength of the aqueous phase had a major influence on reverse micelle extraction.	(Hou et al., 2010)
Small black kidney beans or *Phaseolus vulgaris*	• Liquid-liquid extraction reverse micelle • Anionic surfactant used AOT in isooctane. • Forward extraction with pH in the range of 3.0-10.0, NaCl concentration range of 30-100 mM, AOT concentration within 10-350 mM and extraction time from 5 to 60 min • Backward extraction with fresh aqueous phase, the new 10 mM buffered aqueous phases at different pH (5.0-10.0) and KCl concentration (50-1000 mM)	• Reverse micelle extraction was successfully applied for the extraction and purification of lectin from a crude extract. • Easy to perform with maximum protein recovery 53.28 %.	(He et al., 2013)

2.5.1 Affinity Chromatography

Before affinity chromatography was introduced in 1968, traditional antibody purification protocols relying on salt precipitation, temperature and pH failed to meet the strict quality and regulatory standards required for many biopharmaceutical applications. Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction such as between antigen and antibody, enzyme and substrate, or receptor and ligand. The major materials required for an affinity chromatography procedure are a bead matrix, ligand, solution containing the substrate to be isolated, wash to elute the non-bound impurities in the solution, and a final wash to elute the bound substrate from its ligand. This purification technique requires appropriate matrices as a supporting material for the ligand (Ayyar et al., 2012).

The bead matrix is an agarose gel loaded into an elution column and the matrix should be uniform, macroporous, hydrophilic, chemically as well as mechanically stable, selective, exhibit minimum non-specific absorption, is insoluble in the solvent used in purification, has ideal flow characteristics and provides a large surface area for ligand attachment. Sepharose is the most widely used matrix, because the hydroxyl groups on the sugar residues can be easily manipulated to accept a ligand. Additionally, the matrix must facilitate chemical activation, thus facilitating the coupling of required ligands. However, although initially used extensively for coupling reactions in affinity chromatography, it is highly toxic with attendant safety hazards. A wide range of coupling chemistries, involving primary amines, aldehydes, hydroxyls and carboxylic acids are available for covalently attaching ligands to matrices. Matrices for use in affinity chromatography can be divided into three groups which are natural, synthetic and inorganic. Agarose, dextrose and cellulose beads are commonly employed natural matrices that satisfy the majority of the parameters mentioned earlier. Synthetic supports include acrylamide, polystyrene and polymethacralate derivatives, whereas porous silica and glass are some of the most frequently reported inorganic matrices (Ayyar et al., 2012).

The ligand is then selected according to the desired isolate. Two properties are required for the ligand; firstly is that it binds specifically and reversibly to the isolate, and secondly, that the ligand is capable of covalent bonding to the matrix without disrupting its binding activity. This is usually facilitated by the placement of spacer arms between the ligand and the matrix, so that in case the active site is buried deep within the ligand, it is not physically hidden from its binding substrate. The solution is usually a protein rich mixture such as antiserum, which is poured into the elution column and allowed to run through the gel at a controlled rate. The first wash must be of sufficient salt concentration, pH and temperature to elute all unbound impurities from the solution, but not so extreme that it causes the isolate to dissociate from the ligand. The second wash must elute the isolate, therefore, it must be of suitable concentration, pH, and temperature (Jin et al., 2013).

Affinity chromatography is undoubtedly the most widely employed method for protein purification. Over the past few decades considerable effort has been made to streamline the purification process, in terms of specificity, selectivity, reproducibility, economy, product recovery, storage and maintenance. This was achieved by developing novel affinity methodologies linked to the identification and design of novel ligands and matrices for immobilization. Although it is a highly specific procedure, some carbohydrate matrices can adsorb not only the lectin but also glycosidase that are capable of hydrolysing the sugar structures to which the lectin may bind. Contamination by glycosidases could greatly affect the activity of a lectin preparation. Several types of matrix and ligand are readily available in the market at high prices. In order to obtain high yields and purity it is necessary to consider the type of ligand, the matrix to which it is attached and the purification procedure, which may require optimization depending on the type/class of protein and its ability to recognize the immobilized ligand (Ayyar et al., 2012).

2.5.2 Ion Exchange Chromatography

The most popular method for the purification of proteins and other charged molecules is ion exchange chromatography (IEC). In cation exchange chromatography, positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. The separation of compounds is based on the characteristics of many analytes to enter into specific interactions with certain molecules of the packed material. Electrostatic forces between charged groups, nonpolar interactions, hydrogen bonds and hydrophobic bonds may be involved in the interaction process, in addition to covalent bonding through the use of biospecific ligands (e.g. enzyme substrates, antibodies, receptors) or so-called pseudo-biospecific ligands.

To optimize the binding of all charged molecules, the mobile phase generally has low to medium conductivity and low to medium salt concentration in the solution. The adsorption of molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support. The strength of the interaction is determined by the number and location of the charges on the molecule and on the functional group. By increasing the salt concentration, the molecules with the weakest ionic interactions start to elute from the column first. Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient. The binding capacities of ion exchange resins are relatively high and this is of major importance in process scale chromatography but is not critical for analytical scale separations. Meanwhile during ion exchange chromatography, the crude extracts of jackfruit seeds are extensively dialysed against phosphate buffer and applied onto a column containing DEAE-cellulose (2.5 x 50 cm). The adsorbed protein is eluted by phosphate buffer with a certain concentration and suitable pH, which contains a stepwise gradient of 50, 100, 150, 200 and 500 mM NaCl (Kabir, 1995).

However, some problems arise because proteins have unique properties. Proteins are derived from biological sources and their extraction often requires the use of detergents salts for solubilization. One problem is that such solubilizers interfere with the operation of IEC. Another problem is that proteins in cells are classified and therefore separated from other proteins. Extraction results in disruption of these barriers with proteolysis activity and the exposure of proteins, thus the proteins must be extracted from other biological materials, hence the amount of protein available may be limited, such as when working with embryonic tissue. Another problem is that proteins are ampholytes; that is they contain both positive and negative charges; the former as a result from the ionization of lysine and arginine residue and the latter from aspartic and glutamic acid residue.

2.5.3 Reverse Micelle Extraction Method

The reverse micelles extraction (RME) method has been utilized for extraction and purification of proteins since the 1990s (Anjana et al., 2010). This practice is an alternative to chromatographic techniques, since the system can be easily scaled up and the principle allows for continuous separation processes, similar to the liquid-liquid extraction processes, which are commonly used in the chemical industry. RME technology is based on charge interaction, hydrophobicity and the size of the reverse micelle relative to the droplet. Reverse micelles are able to host the proteins in an aqueous environment, effectively shielding them from a non-miscible one. The RME solvents contain small droplets of water or know as micelle, stabilized within an organic solvent by a surfactant. Protein molecules often move from an original water phase into these small, encapsulated by micelles is attractive for separating proteins from an aqueous solution. This process can be used to separate biological products such as proteins, which have been solubilized in organic solvents using surfactants, without affecting their functional properties. The process can be performed by a forward extraction of the target protein or contaminants, from an aqueous solution to a reversed micelle organic phase, followed by back-extraction, during which the biomolecules are released from micelles and transferred to a new aqueous phase (Anjana et al., 2010).

Micelle in aqueous solution combines with the hydrophilic "head" sections in contact with the surrounding solvent, confiscating the hydrophobic single-tail sections in the micelle center (Figure 2.4). This phase is caused by the storing behavior of single-tail lipids in a bilayer. The difficulty is filling the entire volume of the interior of a bilayer, while accepting the area per head group forced on the molecule by the hydration of the lipid head group, leading to the formation of the micelle. This type of micelle is known as a normal-phase micelle (oil-in-water micelle). Inverse micelles have the head groups at the center with the tails extending out (water-in-oil micelle).

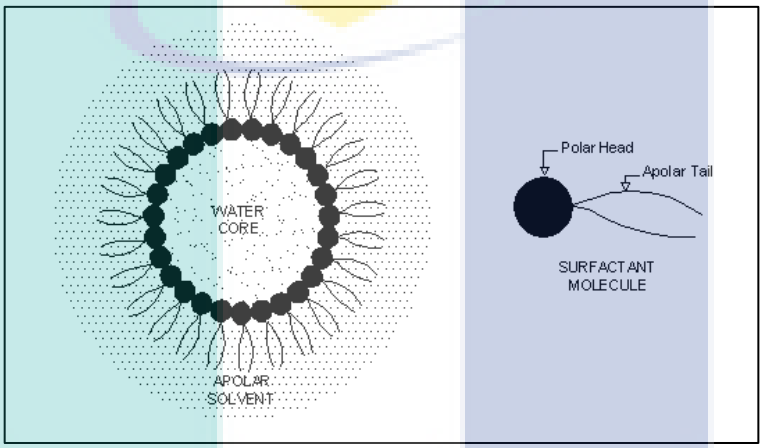


Figure 2.2: Reverse micelle schematic diagram process. Micelles are approximately spherical in shape, although shapes such as ellipsoids, cylinders, and bilayers are also possible.

Source: Hou et al., (2010)

Reverse micelles are thermodynamically stable, nanometer-sized water droplets dispersed in an organic phase by means of surfactant. The size and shape of reverse micelles vary significantly with the nature and the concentration of the surfactant and the solvent used. Size and shape also depend on temperature, pressure, and ionic strength. Reverse micelles have an inner core of water, often called a water pool, which can solubilize hydrophilic biological substances such as DNA, proteins and amino acids. Reverse micelles are used as a reaction system for enzymatic catalysis, liquid–liquid extraction of proteins, and protein refolding in the field of biotechnology. The most researched applications of RME as a protein purification method concerns

hydrolytic enzymes which have the widest variety of uses in food, detergent, textile, pharmaceutical, diagnostics, and fine chemical industries (Basheer and Thenmozhi, 2010). The process of forming micelles is known as micellisation and forms part of the phase behavior of many lipids, according to their polymorphism. The utilization of reverse micelles for lectin extraction has shown that the biomolecules can be extracted from various resources and purified as well as concentrated at the same time, to some extent in one step with a minimal investment in equipment but at a high recovery rate.

Lectin extraction from aqueous solution by this method is a process that utilizes basic techniques of chemical engineering such as classical liquid - liquid extraction, and thus has the potential for industrial application (Anjana et al., 2010 and He et al., 2013). RME is of potentially high industrial importance as it enables one to retain enzymes with high stability and activity. It has yet to be exploited in biotechnological fields of application (Basheer and Thenmozhi, 2010). Nascimento et al., (2002) used reverse micelles to extract lectin from *Cratylia mollis* seeds, of which the extraction and back extraction rate were both 100 %. Extraction and back extraction of reverse micelles is very fast and both can be completed within few minutes. Compared to the tedious operations of traditional extraction methods, the reverse micelles method has been shown to have much greater efficiency. In another study, the efficacy of degreasing with both traditional methods and reverse micelles was investigated, and degreasing was demonstrated to be conducive to the separation of red kidney bean lectin (Hou et al., 2010). RME resulted in a higher extraction rate and higher hemagglutinating activity than the three traditional methods (soaking, degreasing and homogenate), and the protein extraction rate from degreased bean powder with reverse micelles was 31.60 % higher than with the degreasing method. The total hemagglutinating activity of reversed micelles was 99.80 % higher than that of the degreasing method. Therefore, the RME method was considered to be a better method for extracting lectins (Hou et al., 2010). From these comparisons, it can be concluded that reverse micelle purification is the most suitable method for the purification of jacalin from crude jackfruit seed extract. Reverse micelle is now a well-established method for purification of lectins and has become a trend in enzyme technology.

The various advantages have been discussed in numerous papers and the key component, like all chromatographic approaches, is the separation media. Such materials have the advantages of being economical, easily available and non-toxic (so that the separation protocol is compatible with application of the target enzyme in food processing). The use of reverse micelles is thought to be among the most effective due to its high efficiency and selectivity achieved in some systems. This purification method has also been compared with traditional extraction methods and validated to be a time saving method for the extraction of red kidney bean lectin (Hou et al., 2010 and Lee and Chong, 2011).

The utilization of reverse micelles in lectin extraction and purification from crude protein has been successfully applied using an AOT/isooctane/water biphasic system with the addition of butanol on the back-extraction step and adjustment of pH, ionic strength and AOT concentration. Although the maximum recovery (85 %) was found to be at pH 7.0 with 500 mM KCl, the highest purification factor (1.7) was found to be at the same pH level (5.5) found on the extraction step (Nascimento et al., 2008). Furthermore, because the separation process using reversed micelles is based on an ordinary liquid-liquid extraction technique, it is easy to scale up and operate continuously (Lau et al., 2008). However, Table 2.9 showed the summary of extraction of protein from several types of plant seed using liquid-liquid extraction method by reverse micelle extraction

Table 2.9: Summary of extraction of protein from several types of plant seed using liquid-liquid extraction method by reverse micelle extraction

Name of sources	Condition of reverse micelle extraction	Yield	References
Black turtle bean *(Phaseolus vulgaris)*	Forward extraction; AOT surfactant, 77 mM NaCl, pH 5.6, Backward extraction: 593 mM KCI, pH 8.01 63.00 % recovery	1.37 ± 0.12 mg/g sample	(He et al., 2015)
Black kidney bean	Forward extraction; AOT surfactant, 100mM NaCl, pH 5.5, Backward extraction: 500 mM KCI, pH 7 Protein recovery 53.28 %	66.24 mg/g sample	(He et al., 2013)
Red kidney beans lectin	Forward and back extraction; Phase contacted 1:1 (v/v), stirred 5 min, centrifuge at 2292xg for 5 min Protein recovery 12.50 %		(Hou et al., 2010)
Crataeva tapia bark lectin	Forward extraction; AOT surfactant, 30 mM NaCl, pH 5.5, Backward extraction: 500 mM KCI, pH 5.5 Protein recovery 56.00 %		(Nascimento et al., 2008)

2.6 BIOACTIVITY OF CELL LINES

Biological activity or pharmacological activity is described as the beneficial or adverse effects of a drug on living matter. As drug is a complex chemical mixture, this activity is exerted by the substance's active ingredient but can be modified by the other constituents. Among the various properties of chemical compounds, biological activity plays a crucial role since it suggests the uses of these compounds in medical applications. However, chemical compounds may show some adverse and toxic effects which may prevent their use in medical practice. Activity is generally dosage dependent. Furthermore, it is common to have effects ranging from beneficial to adverse for one substance when going from low to high doses.

2.6.1 Cell Culture

Cell culture technology is currently playing an important role in many fields of research, by providing model systems for studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, the process and triggering of aging, nutritional studies, toxicity testing to study the effects of new drugs, studying the function of various chemicals in cancer research, and the use of virus and radiation to convert normal cultured cells to cancerous cells. Cell culture also has an important function when researching the application of jacalin onto cancer cells. In vitro cultivation of organs, tissues and cells at a defined temperature using an incubator, supplemented with a medium containing cell nutrients and growth factors, is collectively known as tissue culture. Different types of cells are grown in a culture, including connective tissue elements such as fibroblasts, skeletal tissue, cardiac, epithelial tissue (liver, breast, skin and kidney) and many different types of tumor cells. Table 2.10 shows previous studies on the application of a variety of seeds on several types of cancer cells. These findings conclude that many seeds may have potential as therapeutic agents for cancer, and jacalin has also been demonstrated to be an effective antigen for human erythroleukemic cancer cells (K562) and colon cancer cells (HT29) (Kabir, 1998). This study will determine the possibility and effectiveness of the application of jacalin on to other cancer cells, for example breast and lung cancer cells.

Table 2.10: Cytotoxicity effects of lectin from various types plant seed towards cancer cell

Seed (Lectin)	Type of cell	Result	References
Jackfruit seed (jacalin)	Human erythroleukemic cancer cells (K562)	Cells express elevated levels of cell surface markers such as CD61 (integrin β 3) and CD14. Jacalin transforms these cells by acting through leukosialin, a heavily O-glycosylated protein	(Kabir, 1998)
Jackfruit seed (jacalin)	Colon cancer cells (HT29)	The inhibition is non-cytotoxic, partial (up to 60 %) and probably mediated through the T antigen, Galβ1–3GalNAcα	(Kabir, 1998)
Salvia bogotensis seed lectin	Tn Antigen	The lectin's specificity and high affinity for the Tn antigen, commonly found in tumour cells, makes this protein a useful tool for immune-histochemical and cellular studies.	(Vega and Pérez, 2006)
Grape seed (proanthocyanidin)	Colon cancer cells	Significantly inhibited cell viability and increased apoptosis in cancer cells, but did not alter viability in the normal colon cell line	(Engelbrecht et al., 2007)
Mangifera pajang (bambangan)	Breast cancer cells (MCF7)	An ethanol extract of the kernel arrested growth of proliferating cells from two breast cancer cell lines.	(Bakar et al., 2010)
Root bark of *Artocarpus elasticus*	Lung cancer cells (A549)	Artonol A exhibited cytotoxic activity against the A549 human cancer cell line, with an IC_{50} value of 1.1 µg/mL	(Jagtap and Bapat, 2010)
Citrus	Pulmonary, colon and breast cancer cell	Inhibits cells based on cell culture and animal studies by D-Limonene, a major monoterpene in citrus	(Dembitsky et al., 2011)
Grape seed	Skin cancer cell	Proanthocyanidins have the ability to reactivate or restore the expression of the DNA hypermethylation-silenced tumor human skin cancer cells	(Vaid et al., 2012)
Bitter gourd *(Momordica charantia)* seeds	Liver cancer cells	Natural component from *Momordica charantia* is MAP30. Medicinal applications of MAP30: one is the application to fight against HIV and the other is its tremendous therapeutic promise on tumors and latter scenario merits clinical trials in the management of liver cancer.	(Fang et al., 2012)
Lemon seed	Breast cancer cells (MCF7)	Bioactive components in lemon seed extracts could be a good source of antioxidants and induce apoptosis in MCF7 breast cancer cells through the mitochondrial apoptosis pathway	(Kim et al, 2012)
L. Satvium seed	Breast cancer cells (MCF7)	Effective against MCF7 cells compared to human skin fibroblast (HFS) cells. In general, the highest (75 %) dose of extract was as cytotoxic for both MCF7 and HFS cells in most assays.	(Mahassni and Reemi, 2013)
Adlay seed *(Coix lachryma-jobi L.)*	Human non-small lung cancer cells (A549)	Demonstrate that polysaccharide fraction is capable of inhibiting A549 cell proliferation and inducing apoptosis.	(Lu et al., 2013)

2.7 OPTIMIZATION STRATEGIES

Optimization is referred to as a way to improve the performance of a process, product or system in order to achieve the maximum benefit from it. Generally, the term 'optimization' has been used in analytical chemistry as a means of discovering conditions applicable to a procedure that generate the best possible response. In this study, factors that significantly affect the jacalin production were screened by Design of Expert (DOE); the one-factor-at-a-time (OFAT) approach to obtain the most optimum possible level for all the factors, and the optimization process by response surface methodology (RSM), so that the relationships between the factors and the response variables could be determined (Salihu et al., 2011). In order to scale-up the biochemical jacalin production, the process should be optimized. Commonly used optimization techniques are briefly described below.

2.7.1 One-Factor-At-Time Approach

The OFAT or One-Factor-At-Time approach method optimization in analytical chemistry has been carried out by monitoring the influence of one variable at a time on an experimental response. While only one process variable is changed, others are maintained at a constant value. It is the most extensively used experimental strategy for process optimization (Tinoi et al., 2005). In an OFAT approach, a researcher pursues information about one parameter in each experimental trial and this procedure is repeated in turn for all factors to be investigated (Frey et al., 2003). This classical method is time consuming and incapable of detecting the true optimum conditions, especially due to the absence of studying the interaction effects among the factors (Bezerra et al., 2008). Meanwhile, statistically designed experiments that vary several factors simultaneously are more efficient when studying two or more factors aiming for optimization. However, the advantages of statistically designed experiments include reducing the number of experiments done, requiring less number of resources, offering more precise estimation of results as more observations are used in determining the effect of each factor (Abdel-Hafez et al., 2014).

Statistical optimization methods are successfully used to identify the optimal level of various parameters involved in the process. Optimization by changing one factor at a time was a common and well-studied method but it has many disadvantages such as being time consuming, expensive not providing details about the interaction effects of the variables involved in the process (Kumar et al., 2013). Another disadvantage of the one-factor optimization is the increase in the number of experiments necessary to conduct the research, which leads to an increase in time and expenses as well as an increase in the consumption of reagents and materials (Bezerra et al., 2008). Therefore, to mitigate this issue, a few carefully selected and simple OFAT variations showing influence upon these responses are performed, in order to obtain additional information regarding the factors of influence and responses of interest, estimate the cut point levels, approximately define the space of possible factor settings as well as adjust the experimental plan and avoid unnecessary experiments during the subsequent optimization process (Simonoska et al., 2013). Nevertheless, a limited number of factors are always studied whether in OFAT experiments or in experiments designed statistically. In order to overcome this problem, the optimization of analytical procedures has been carried out by using multivariate statistic techniques. Among the most relevant multivariate techniques used in analytical optimization is RSM (Bezerra et al., 2008).

OFAT and RSM approach in optimization design experiments have been used for many studies, including optimization of the enzymatic hydrolysis parameters for preparing antioxidant peptides by trypsin and pepsin (Rafiqul and Mimi Sakinah, 2012 and Lin et al., 2013), investigating the effects of pulsed electric field treatment parameters on antioxidant activity of polypeptides (Wang et al., 2012), enhancing the production of lipase by candida cylindracea using palm oil mill effluent as a basal medium in shake flask cultures (Salihu et al., 2011), determining the key formulation factors (concentration and volume of drug solution, evaporation rate and ratio) influencing nanoparticle properties (particle size and size distribution, encapsulation efficiency, drug content, zeta potential, drug dissolution rate, as well as protein binding capacity) (Simonoska et al., 2013) and also in modeling chitosan nanoparticles production (Abdel-Hafez et al., 2014).

2.7.2 Response Surface Methodology

Determination of the effects of interactions among the variables, in addition to the optimization of the production process, has been carried out using multivariate statistical techniques. Among those techniques, RSM is the most relevant method used in optimization studies (Bezerra et al., 2008). This term originated from the graphical perspective generated after fitness of the mathematical model, and its use has been widely adopted in texts on chemo metrics. RSM consists of a group of mathematical and statistical techniques that are based on the fitness of empirical models to the experimental data obtained in relation to experimental design.

Several stages in the application of RSM as an optimization technique are as follows: (1) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; (2) the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fitness of a polynomial function; (4) the evaluation of the model's fitness; (5) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (6) obtaining the optimum values for each studied variable (Bezerra et al., 2008). RSM is a collection of statistical tools used to analyze and determine the optimal conditions within the design space of the experimental study. The main advantage of using RSM is to understand the interaction among the process variables with fewer experimental runs and it has been utilized well for various optimization studies (Kumar et al., 2013).

Numerous statistical designs for RSM have been reported, such as CCD (Central Composite Design), BBD (Box-Behnken statistical experiment design) and two-level FFD (full factorial design). However, CCD remains the most extensively used technique in application (Dutta et al., 2014). It is usually used to study and build a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. After the design experiment has been performed, linear regression is used to obtain results. The present study attempts to assess CCD

optimization technique embedded in RSM through three major steps comprising of statistically designed experiments performance, estimation of the coefficients in a mathematical model through regression and prediction of response as well as validation of the model. The total number of experimental runs depends on the number of parameters explored in the process and is associated with factorial, axial and replicate runs as given by the following expression:

$$N = 2^n + 2n + n_c \tag{2.1}$$

where n is the number of independent variables and n_c is the number of replicate runs. The CCD was found to be a valuable tool for estimating the effects of the extracting pressure, extracting temperature, flow rate of carbon dioxide and their interactions for the purpose of optimizing the extraction of tocopherols from kalahari melon and roselle seeds (Nyam et al., 2010). Kumar et al., (2013) also applied CCD to studied at five levels and evaluate the optimal conditions of three process variables, namely mass of sample, extraction time and temperature.

2.8 CONCLUSIONS

Jacalins are plant lectins containing in jackfruit seed, which is an easily obtained source in Malaysia. It is special lectins in the pharmaceutical and has been proven to be useful as an antibacterial, therapeutic agent and as inhibitor for most human carcinomas. There are many studies only focuses on the application and function of jacalin but are inattentive on the optimization of recovery and purification processes. Currently purification of jacalin using chromatography methods which is involve high costs in order to prepare the suitable column and materials to be scaled up for commercial industries. Therefore, this study solves these problems with optimization of recovery of crude protein and applied liquid-liquid extraction by reversed micelles was used as alternative methods to replace the purification of jacalin.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

The bulk of the research works for this thesis consist of experimental laboratory activities, namely sample preparation, pretreatment process, recovery of crude protein, extraction of jacalin and cytotoxicity study on cancer cells. The optimum conditions for crude protein recovery plays an important role in the overall extraction yield of jacalin, hence this research was focused on investigating the optimum conditions of crude protein recovery from samples using One-Factorial-At-Time (OFAT) and Response Surface Methodology (RSM) based on liquid-liquid separation, reverse micelle extraction of jacalin from crude protein.

Figure 3.1 shows the overall block diagram of the experimental works that were carried out. The initial stage of the study examined the pretreatment time that resulted in the highest crude protein concentration. The samples were pretreated and went through crude protein recovery. Upon the recovery of crude protein; experiments were carried out to optimize the operating conditions of recovery to maximize the yield. The optimum operating conditions of the recovery process were then employed during the process of extracting jacalin from the crude protein. The collected jacalin was then applied cytotoxicity an *in vitro* study to examine the potential of jacalin as a cancer therapy agent for two types of cancer cells, namely human breast cancer (MCF7) and non-small lung carcinomas (H1299).

3.2 MATERIALS

3.2.1 Chemicals and Reagents

All chemicals used in the study were of analytical grade and purchased from various suppliers. A list of the chemicals used in this study is given in Appendix A. Ultrapure water was used to prepare various solutions and reagents.

3.2.2 Raw Material (Jackfruit Seed)

The jackfruit seeds used throughout this study were collected directly from fresh fruits of the jackfruit species *Madu Mastura*. They were bought from a jackfruit farm in Temerloh, a place mostly free from industrial pollution to ensure the fruits and seeds were healthy. This species is easily found species in Malaysia because of high demand since 2000 (Zakariya, 2014).

3.2.3 Cell Culture

Two human cancer cell lines, MCF7 (human breast cancer) and H1299 (human non-small lung carcinoma), were kindly provided by Dr. Masa-aki Ikeda; Department of Molecular and Carniofacial Embryology, Tokyo Medical and Dental University.

3.3 OPERATIONAL FRAMEWORK

Figure 3.1 presents the experimental flowchart for this study. Flowchart was starting preparation of jackfruit seed powder sample, followed by pretreatment process, recovery of crude protein, extraction of jacalin from crude protein and last part is cytotoxicity study on cancer cell. Meanwhile Figure 3.2 showed the operational frame work of overall this sudy. The methodology was divided into four part; characterization, crude protein recovery, extraction of jacalin by reverse micelle and cell culture and cytotoxicity study.

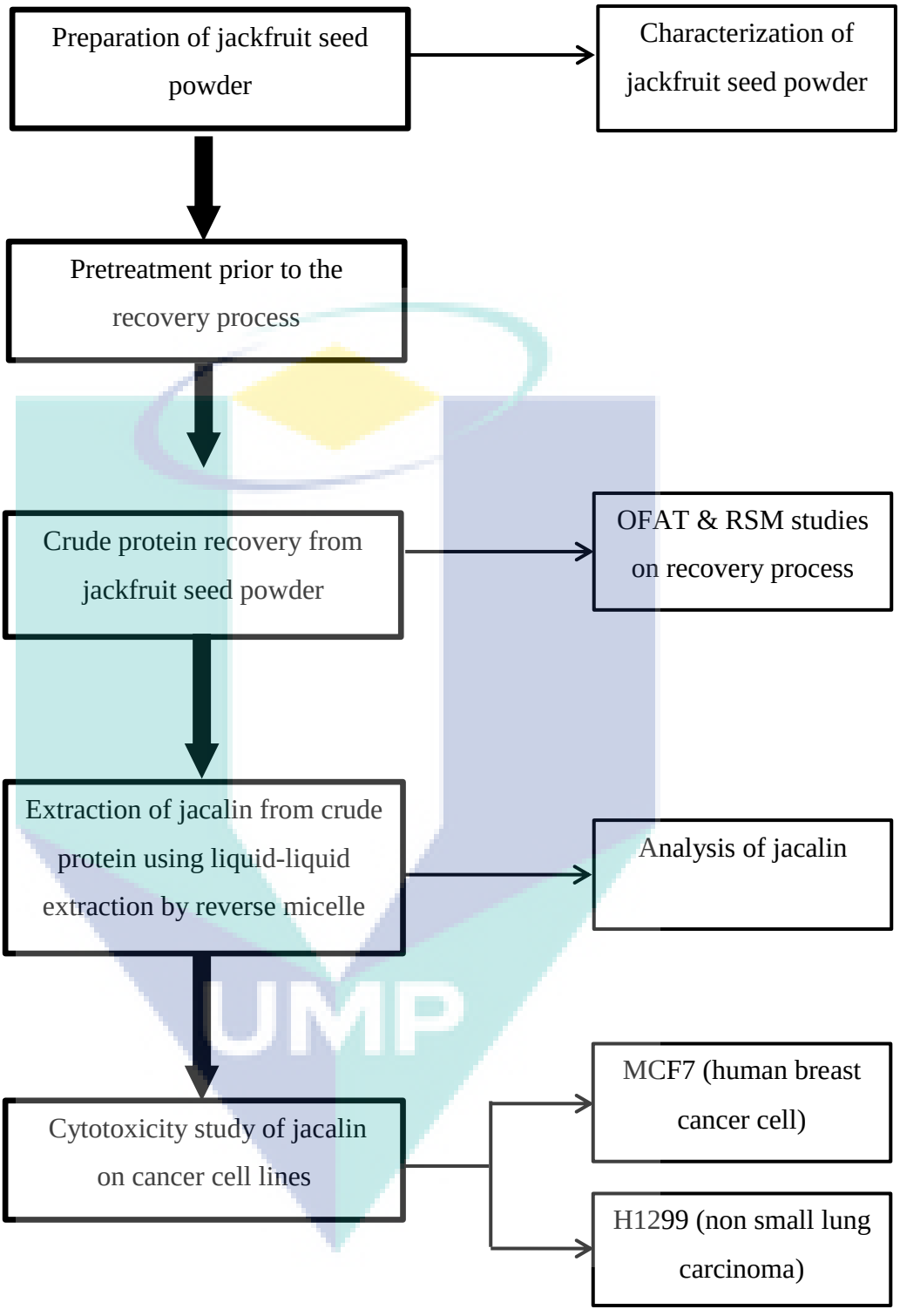


Figure 3.1: Experimental flowchart for this research

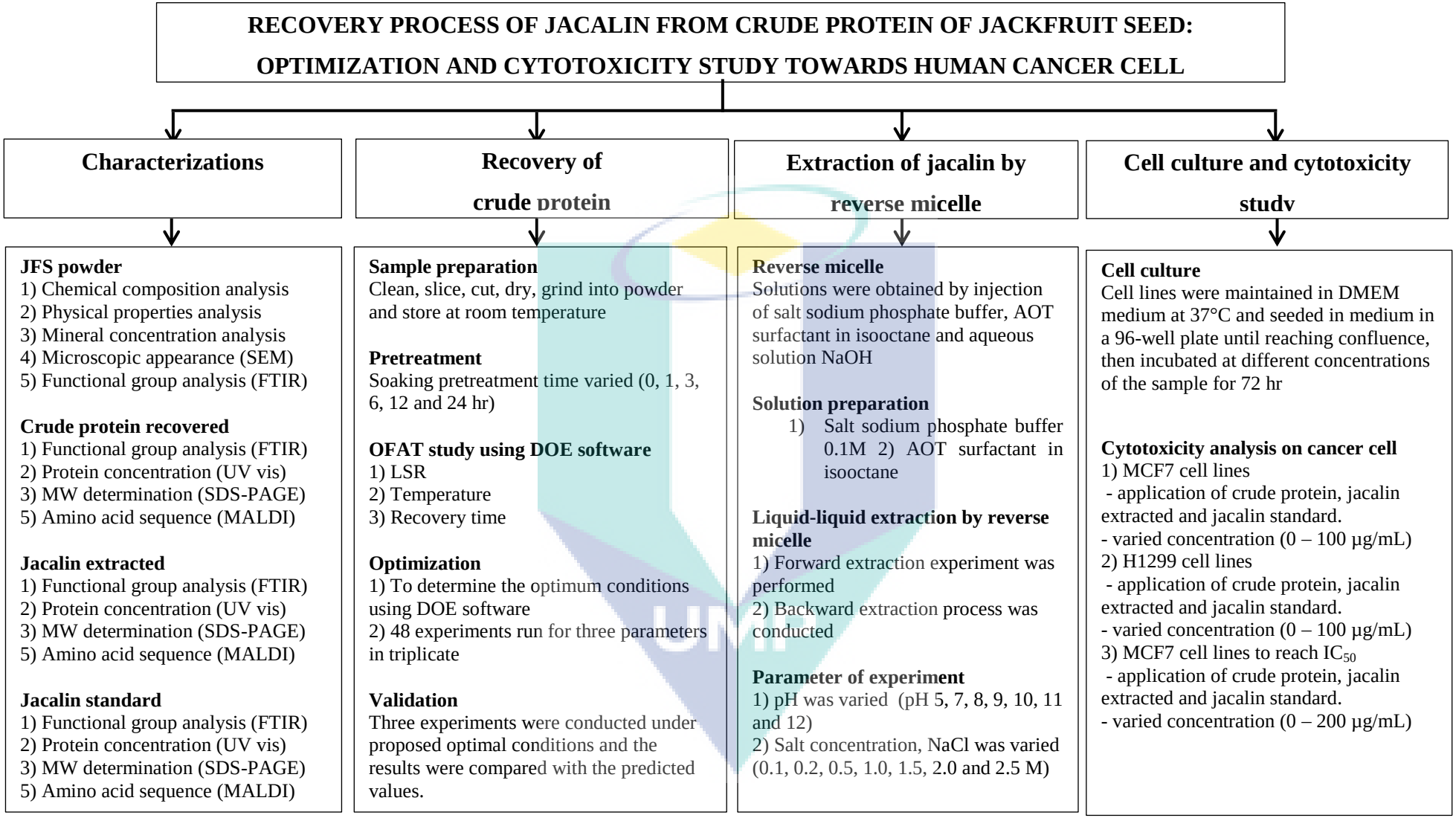


Figure 3.2: The operational frame work of this research

3.3.1 Preparation of Jackfruit Seed Powder

The jackfruit seed contributes up to 15 % of the total fruit weight. There are many species of jackfruit called *nangka madu*, such as *nangka madu CJ1, CJ2, CJ3, CJ4, CJ5, CJ6* and *Mastura.* However, *nangka madu Mastura* (Figure 3.3) was selected as the raw material in this study since it is easy to obtain, highly in demand and is a nonseasonal species. The jackfruit was obtained from an entrepreneur in Temerloh, Pahang at a price of RM1.70 per kg. The *nangka madu Masturas* species from in same batch, same season and same location was used for this study to maximize the accuracy of finding in this study.

The fruit has latex in the epidermis and the seeds are coated by a thin whitish membrane (1 mm in thickness). Thus, the seeds had to be carefully separated from the flesh to avoid having any sticky latex attached to the seeds. Then, the removed seeds were cleaned and their whitish membrane coats were peeled off. The cleaned seeds were firstly cut into slices of seed chips with around 2 to 5 mm thickness, in order to increase their surface area and to make the drying process easier (Tulyathan et al., 2002). Figure 3.4 (a), (b), (c) and (d) showed the image of seed coated with whitish membrane, peeled off membrane, peeled seed and sliced seed. The purpose of the drying process was to ensure consistency during sample weighing and avoid any moist contamination in the sample. The drying process was done through direct sun drying without removing the thin brown spermoderm covering the fleshy white cotyledons. The sample was subjected to continuous drying, in which the sample was weighed before and after drying under the sun every day, until there was no further change in the weight of the sample. After 7 days of continuous drying, the average weight of the sample was found to be reduced by 60 %, equivalent to 2.29 kg compared to 6.23 kg before drying. This once prepared powder was used throughout the study until completion.

Figure 3.3: Jackfruit M*adu Mastura* with seeds

Figure 3.4: (a) The seeds coated with whitish membrane (b) The peeled off whitish membrane coat (c) The peeled seeds and (d) Sliced chips of jackfruit seeds

After drying, the samples were grind into powder using a mixer-grinder, brand Retsch model ZM200, with 550 watts and 17000 rpm rotating speed electrical motor. The seeds were grinded well uniformly for 10 min and turned into particle-sized powder (<0.5 mm). The grounded sample was then passed through a sieving process to measure its particle size distribution using a vibratory sieve shaker Analysette 3 Pro, Fritsch, Germany to select the particles with less than 0.5 mm (>0.5mm retained) size. Sieve shaker is the most widely used method for analysis of aggregate or particles larger than

50 µm. In some cases, sieves can be used for fine particle analysis (down to 5 µm). The sieving method is based only on the size of the particle and is independent of other particle properties (e.g., density, optical properties and surface roughness). The common sieves are made of woven cloth and have square apertures. The sizes of the sieve openings have been standardized and currently two different sets of standard series, the Tyler Standard and the U.S Series ASTM standard, are used in the United States (Zuraidah, 2010). The average particle size was calculated using the Tyler method and identified to be 0.335 mm. In the recovery process, the rate of recovery was found to be increased when the area of contact between the liquid and solid was high. The sample powder materials were packed in plastic pouches and stored in a refrigerator until use (Bhat and Pattabiraman, 1989 and Tulyathan et al., 2002).

3.3.2 Soaking Pretreatment Experimental Work

The pretreatment soaking time of jackfruit seed powder was varied in order to determine the optimum soaking time prior to performing the recovery process. Six variations of soaking time within 0 to 24 hr were studied using a conventional extraction method that employed phosphate buffered saline (PBS), precipitated and filtrated at fixed operating conditions (PBS, 0.1 M, pH 7.4, 250 mL, 4 ^{o}C). Then, 10 g of the sample was pretreated at varied soaking times with a liquid to solid ratio (LSR) of 3 mL/g, followed by the recovery process. The steps in the determination of the crude protein and jacalin are illustrated in Figure 3.1 and the matrix for the soaking pretreatment experiments is shown in Table 3.1. All the experiments were conducted in solutions of PBS at pH 7.4 and concentration of 0.1 M, as mentioned in previous studies (Kabir, 1995).

Experiment A1 (without pretreatment) was conducted as the control experiment, in which crude protein was used without any soaking pretreatment. Experiment A6 (24 hr soaking time) was conducted to provide a comparison with crude protein pretreatments from previous studies by Kabir (1995) and Roy et al (2005). Hence, experiment A2 to A6 was conducted to determine the actual optimum soaking pretreatment time for crude protein of jackfruit seed powder. The pretreatment times were varied to study the effect of soaking pretreatment in the recovery process later.

Table 3.1: Experimental matrix for soaking pretreatment variables

Experiment	Soaking Time (hrs)	Temperature (^{o}C)	Volume of PBS (mL)	Mass Sample (g)	Recovery Time (hr)
A1	0	4	30	10	5
A2	1	4	30	10	5
A3	3	4	30	10	5
A4	6	4	30	10	5
A5	12	4	30	10	5
A6	24	4	30	10	5

3.3.3 Crude Protein Recovery from Jackfruit Seed Powder

The crude protein recovery experiments were carried out by PBS precipitation followed by centrifugation and filtration. The optimum time of soaking pretreatment was applied for all the recovery processes of crude protein. The conditions for the use of PBS were fixed, and PBS was prepared as mentioned in the previous section. Liquid to solid ratio (LSR), temperature and recovery time were varied to study the performance of the recovery process. The volume of PBS to sample ratio was varied in the range from 3 to 15 v/w (mL/g), while temperature was varied in the range 4 to 40 ^{o}C and the duration of the recovery process was varied in the range of 0 to 12 hr.

The following represents the typical recovery procedure method as was used in this study: The sample was mixed with 0.1 M PBS with pH 7.4 at 4 °C and was soaked as in the pretreatment step (as detailed in 3.3.2). The pretreated sample was then incubated at 4 ^{o}C with constant shaking at 150 rpm for a specified time period in an incubator shaker (Kuhner Model Lab Therm LT-X). All the samples were then centrifuged at 10,000 rpm for 20 min using Eppendorf Centrifuge 5810 R. The clear supernatant was passed through a millipore filter paper (0.45 µm) and the crude protein concentration was measured by the Varian's Cary 50 UV-Vis Spectrometer and calculation of absorbance was done using the Lowry method. The process is outlined below for all the experiments conducted as shown in Table 3.2, as based on Design of Expert (DOE) software using One Factorial Design or One-Factor-At-Time (OFAT).

a) Design Parameters of One-Factor-At-Time

i) Liquid to solid ratio (LSR)

The range of LSR in this study was from 3 to 15 mL/g at a constant recovery time of 3 hr and temperature of 4 ^{o}C. 10 g of sample JSP was mixed with various volume of PBS within 30 to 150 mL. The range of ratio was referred and modified from previous studies by Kabir (1995) and Roy et al. (2005).

ii) Temperature

The temperature of the recovery process was varied between 4 to 40 ^{o}C in order to determine the optimum temperature for the recovery of crude protein, with a constant 3 hr recovery time and 6 mL/g of LSR. The maximum range of the temperature was based on denature temperature of protein and also denoted to previous study by Kabir (1995).

iii) Recovery time

The experiment was performed within 0 to 12 hr of pretreated sample for determination of the optimum period, with a constant temperature of 4 ^{o}C and 6 mL/g of LSR. The range of the recovery time was modified from previous studies (Kabir, 1995; Roy et al., 2005 and Uraya et al., 2011)

Table 3.2: Variation of process factors for LSR using OFAT

Run Order	Standard Order	Block	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)
1	6	Block 1	12.00	4.00	3.00
2	3	Block 1	6.00	4.00	3.00
3	8	Block 1	15.00	4.00	3.00
4	5	Block 1	9.00	4.00	3.00
5	1	Block 1	3.00	4.00	3.00
6	7	Block 1	15.00	4.00	3.00
7	4	Block 1	9.00	4.00	3.00
8	2	Block 1	3.00	4.00	3.00

Table 3.3: Variation of process factors for temperature using OFAT

Run Order	Standard Order	Block	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)
1	6	Block 1	6.00	31.00	3.00
2	3	Block 1	6.00	13.00	3.00
3	4	Block 1	6.00	22.00	3.00
4	7	Block 1	6.00	40.00	3.00
5	5	Block 1	6.00	22.00	3.00
6	8	Block 1	6.00	40.00	3.00
7	1	Block 1	6.00	4.00	3.00
8	2	Block 1	6.00	4.00	3.00

Table 3.4: Variation of process factors for recovery time using OFAT

Run Order	Standard Order	Block	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)
1	6	Block 1	6.00	4.00	0.00
2	3	Block 1	6.00	4.00	6.00
3	8	Block 1	6.00	4.00	0.00
4	5	Block 1	6.00	4.00	3.00
5	1	Block 1	6.00	4.00	12.00
6	7	Block 1	6.00	4.00	6.00
7	4	Block 1	6.00	4.00	9.00
8	2	Block 1	6.00	4.00	12.00

Table 3.5: Low and high value of process factors for crude protein recovery

Independent Variables	Level		
	Codified	Low	High
LSR (mL/g)	*A*	2.50	3.00
Recovery time (hr)	*B*	3.00	4.00
Temperature (^{o}C)	*C*	25.00	28.00

The significant range effects of LSR, temperature and recovery time for the recovery of crude protein from JSP were selected through the one-factor-at-a-time (OFAT) method. The range as determined by OFAT was then used in the following step, which is the optimization of the recovery of crude protein by applying RSM. Central Composite Design (CCD) was applied in the optimization of crude protein

recovery from jackfruit seeds. The interaction between the parameters could also be evaluated. Factorial design was employed to define the important factors and determine the interrelationships among LSR, temperature and recovery time in enhancing crude protein yield. In theory, the CCD consists of 2n factorial runs with 2n axial runs and center runs, depends on how many factors:

$$N = 2^n + 2n + n_c \quad (2.1)$$

where N is the total number of experiments required, n is the number of factors in the CCD, and c is the center point of the experiment, which was 6 in this study. In this study, the dependent variables selected for the CCD study were: (A) the LSR; (B) the temperature recovery process and (C) the recovery time. The central point was replicated three times to find the system error, and thus there was no need for replication of the entire experiment (Xu et. al., 2014). The CCD experimental works as shown in Table 3.6 were carried out in a randomized order.

b) Design Parameter for Optimization of Crude Protein Recovery

Table 3.6: Variation of process factors for optimization using CCD

Run Order	Standard Order	Block	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^oC)	Factor 3 C:Time (hr)
1	20	Block 1	2.50	31.00	5.00
2	35	Block 1	3.00	28.00	6.00
3	42	Block 1	3.00	34.00	4.00
4	43	Block 1	3.00	28.00	4.00
5	12	Block 1	3.50	25.00	5.00
6	9	Block 1	2.50	25.00	5.00
7	21	Block 1	2.50	31.00	5.00
8	16	Block 1	3.50	31.00	3.00
9	4	Block 1	3.50	25.00	3.00
10	29	Block 1	4.00	28.00	4.00
11	25	Block 1	2.00	28.00	4.00
12	24	Block 1	3.50	31.00	5.00
13	46	Block 1	300	28.00	4.00
14	47	Block 1	3.00	28.00	4.00
15	26	Block 1	2.00	28.00	4.00
16	30	Block 1	4.00	28.00	4.00

Table 3.6: Continued

Run Order	Standard Order	Block	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)
17	45	Block 1	3.00	28.00	4.00
18	19	Block 1	2.50	31.00	5.00
19	8	Block 1	2.50	25.00	5.00
20	6	Block 1	3.50	25.00	3.00
21	23	Block 1	3.50	31.00	5.00
22	39	Block 1	3.00	22.00	4.00
23	48	Block 1	3.00	28.00	4.00
24	10	Block 1	3.50	25.00	5.00
25	13	Block 1	2.50	31.00	3.00
26	11	Block 1	3.50	25.00	5.00
27	2	Block 1	2.50	25.00	3.00
28	33	Block 1	3.00	28.00	2.00
29	3	Block 1	2.50	25.00	3.00
30	32	Block 1	3.00	28.00	2.00
31	28	Block 1	4.00	28.00	4.00
32	27	Block 1	2.00	28.00	4.00
33	7	Block 1	2.50	25.00	5.00
34	31	Block 1	3.00	28.00	2.00
35	34	Block 1	3.00	28.00	6.00
36	15	Block 1	2.50	31.00	3.00
37	44	Block 1	3.00	28.00	4.00
38	18	Block 1	3.50	31.00	3.00
39	40	Block 1	3.00	34.00	4.00
40	38	Block 1	3.00	22.00	4.00
41	5	Block 1	3.50	25.00	3.00
42	1	Block 1	2.50	25.00	3.00
43	22	Block 1	3.50	31.00	5.00
44	17	Block 1	3.50	31.00	3.00
45	36	Block 1	3.00	28.00	6.00
46	41	Block 1	3.00	34.00	4.00
47	37	Block 1	3.00	22.00	4.00
48	14	Block 1	2.50	31.00	3.00

Table 3.7: Low and high value of process factors for optimization using CCD

Independent Variables	Level		
	Codified	Low	High
LSR (mL/g)	*A*	2.00	4.00
Recovery time (hr)	*B*	2.00	6.00
Temperature (^{o}C)	*C*	22.00	34.00

3.3.4 Extraction of Jacalin using Reverse Micelle Method

Liquid-liquid extraction by reverse micelle was performed to assess its capability of producing high concentrations of jacalin. The assessment of reverse micelle extraction for the extraction of jacalin from crude protein was performed at optimum LSR, temperature and recovery time, which determined in the previous experiment. The reverse micelle solutions were obtained through the injection of equal volumes of salt sodium phosphate buffer, stock solutions of the AOT surfactant in isooctane and aqueous solution sodium hydroxide (NaOH). Salt sodium phosphate buffer with 0.1 M and stock solutions of the AOT surfactant in isooctane were prepared as mentioned in section 3.3.4 (a). The reagent stock solution was adjusted to the necessary pH with added 0.1 M NaOH or 0.1 M acid chloride (HCI). The mixtures were then shaking in incubator shaker for 30 min (a completely transparent solution was obtained, which confirms the formation of reverse micelles). The final reagent stock of reverse micelle solutions was prepared for use during the extraction of jacalin.

The forward extraction experiment was performed by mixing 15 mL of crude protein with an equal volume of reverse micelle solution at the desired pH in a 50 mL conical flask. A mixing speed of 400 rpm in an incubator shaker was used in all experiments. The flask was covered to prevent loss of solution by splashing or evaporation. Mixing was carried out for 30 min. The dispersion was then centrifuged at 3000 rpm for 10 min to obtain a distinct phase boundary and the separated phases were examined to determine their protein concentrations using absorption measurement (Lau et al., 2008; Setapar et al., 2012 and He et al., 2013). The pH before and after mixing was measured as initial pH and equilibrium pH, respectively. The percentage of extraction was used to represent the transfer efficiency of jacalin from the aqueous phase to the reverse micelle phase, which is defined as the ratio of the amount of jacalin extracted into the reverse micelle phase to the total amount of the protein present initially (Lau et al., 2008).

The backward extraction process was conducted by adding an equal volume of the reverse micelle phase containing proteins from the forward transfer (stripping solution) and the fresh aqueous phase (0.5 M KCl) with a fixed pH of 7 (neutral) in an

50 mL conical flask and shaking at 400 rpm speed in an incubator shaker. The two phases were mixed for 30 min. Then, the mixture was centrifuged for 10 min at 3000 rpm. The jacalin concentration was determined by absorption measurement at 750 nm for protein using an UV-Vis by Shimadzu Co. All the experiments were carried out at 25.0 ± 1.0 °C. The jacalin concentration in the reverse micelles phase was also determined using mass balance. The transfer efficiency of the backward transfer is defined as the ratio of the amount of jacalin extracted into the buffered aqueous phase to the initial amount of crude protein loaded in the reverse micelle phase from the forward transfer.

Time-based experiments were carried out for the forward experiments to study the kinetic portioning effects of jacalin. While mixing, 1 mL of the sample was taken out at 5 min time intervals. The procedure is outlined below, and the experimental work was carried out following the matrix as shown in Table 3.6.

a) Preparation of solutions for reverse micelle extraction

i) Preparation of AOT in isooctane

Sodium di-2-ethylhexylsulfoccinate (AOT) was chosen as a surfactant in this study for the preparation of the reverse micelle phase. Thus, 0.1 M AOT in isooctane was formulated by mixing 4.45 g of AOT with 100 mL of isooctane to perform all the experiments. In order to assure solution stability, the stock solutions were prepared at least 1 day before use (Jiménez et al., 2002; Setapar, 2008 and Lee and Chong, 2011).

ii) Preparation of salt solution buffer

Sodium chloride (NaCl) was selected for the preparation of a salt aqueous solution for reverse micelle performance this study. Therefore, 0.2 M NaCl was prepared by diluting 1.17 g of NaCl in 100 mL of ultrapure water.

iii) Preparation of KCl solution for backward extraction

9.32 g of KCl was diluted in 250 mL of ultrapure water to prepare 0.5 M KCl aqueous solution.

iv) Preparation of aqueous solution

1 g of NaOH was diluted in 250 mL of ultrapure water to prepare 0.1 M NaOH aqueous solution. The solution was prepared at different pHs in range of pH 5 to 12 by adding NaOH or HCl, based on the experimental matrix shown in Table 3.8.

b) Design Parameters of Reverse Micelle (Forward) Extraction

i) pH of reverse micelle solution

The pH of the reverse micelle solution was adjusted by adding drop of NaOH and HCl. The pH was varied between pH 5 to 12, based on previous research (Jiménez et al., 2002; Lau et al., 2008 and Setapar et al., 2012).

ii) Salt concentration, NaCl

The concentration of NaCl was varied between 0.1 to 2.50 M. The preparation of NaCl was carried out with adjustment of concentration as desired (Anjana et al., 2010 and Hou et al., 2010).

Table 3.8: Experimental matrix for forward extraction by reverse micelle variables

Variables	Experiment	pH	Salt Concentration (M)
Salt concentration	B1	6-7	0.10
	B2	6-7	0.20
	B3	6-7	0.50
	B4	6-7	1.00
	B5	6-7	1.50
	B6	6-7	2.00
	B7	6-7	2.50
pH value	C1	5	0.20
	C2	6	0.20
	C3	7	0.20
	C4	8	0.20
	C5	9	0.20
	C6	10	0.20
	C7	11	0.20
	C8	12	0.20

3.3.5 Application of Jacalin on Cancer Cell Lines

Jacalin that had been extracted from crude protein was applied to two human cancer cell lines to study the effectives of jacalin in reducing the viability of cancer cells. This was performed through *in vitro* study consisting of 96-well plates and examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MCF7 (human breast cancer) and H1299 (non-small lung carcinoma) cell lines that were used in this study were kindly provided by Dr. Masa-aki Ikeda; Department of Molecular and Carniofacial Embryology, Tokyo Medical and Dental University. The cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and glutamine supplemented with 10 % heat inactivated fetal bovine serum (FBS) and 1 % penicillin streptomycin, at 37 °C in a humidified atmosphere containing 5 % CO_2. The MTT assay was used to evaluate the antiproliferative activities of the protein against the cancer cell lines. The assay depends on the cleavage of tetrazolium salt into formazan blue by the mitochondrial enzyme succinate dehydrogenase. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of viable cells present. Thus, the MTT assay is potentially useful for assaying both cell viability and the antiproliferative activities of materials.

For this purpose, cells were seeded in complete medium in a 96-well plate at a density of 1 x 10^5 cells/mL and incubated for 24 hr or until cells confluence. In another 96-well plate, a series of sample dilutions were prepared in DMEM ranging within 0 to 200 µg/mL. When the cells were confluence, medium was taken out and the cells were washed twice with phosphate buffer solution (PBS) before replacing with new medium of DMEM to ensure complete of nutrition supplied to cell along the treatment conducted. Then, the cells were treated in triplicate in the same 96-well plate with different concentrations of samples and were incubated for 72 hr. The medium was then discarded and the adherent cells were washed twice with phosphate buffer solution (PBS), then 20 µL of MTT stock solution (5 mg/mL in PBS) were added to each well and the plates were further incubated for 4 hr at 37 °C. Next, 100 µL of dimethylsufoxide (DMSO) was added to each well to solubilize the formazan crystals produced by viable cells. After formazan blue was completely dissolved, absorbance

was measured at 570 nm wavelength, using TECAN infinite M200 microplate reader. The details of the experimental work of studying the cytotoxicity of the cell lines are shown in Table 3.7, 3.8 and 3.9.

a) Design parameter for cytotoxicity study on cancer cell.

Table 3.9: Experimental matrix of cytotoxicity analysis on cancer cell H1299

Variables	Experiment	Concentration (µg/mL)
Crude Protein	G1	50.00
	G2	100.00
Jacalin Extracted	H1	50.00
	H2	100.00
Jacalin Standard	I1	50.00
	I2	100.00

Table 3.10: Experimental matrix of cytotoxicity analysis on cancer cell MCF7

Variables	Experiment	Concentration (µg/mL)
Crude Protein	D1	50.00
	D2	100.00
	D3	200.00
Jacalin Extracted	E1	50.00
	E2	100.00
	E3	200.00
Jacalin Standard	F1	50.00
	F2	100.00
	F3	200.00

3.4 CHARACTERIZATION JACKFRUIT SEED

The analysis of jackfruit seed powder (JSP) composition is very important to assess the nutrition in lectin (jacalin). JSP characterization was performed via chemical analysis of its structural components. The major constituents of JSP nutrition were analyzed through standard methods as detailed below according to AOAC (1985) methods (Ali et al., 2009). The seed powder was analysed for proximate composition in percentage. All of the analysis was done in triplicate in moisture free conditions, except for analysis of crude protein content.

3.4.1 Determination of Moisture Content

5 g of powder seed sample was weighed into a previously weighed moisture cup and dried in an oven at 60 ^{o}C until a constant weight was reached (Airani, 2007).

$$\text{Moisture \%} = \frac{Initial\ weight\ (g) - Final\ weight\ (g)}{Sample\ weight\ (g)}\ x\ 100 \qquad (3.1)$$

3.4.2 Determination of Ash

The amount of ash was determined by igniting samples in a muffled furnace at 600 ^{o}C for 3 to 4 hrs (Airani, 2007). The amount of ash was expressed as a percentage of the total weight of the sample.

$$\text{Total ash \%} = \frac{Weight\ of\ crucible\ with\ ash\ (g)}{Weight\ of\ crucible\ with\ sample\ (g)}\ x\ 100 \qquad (3.2)$$

3.4.3 Determination of Crude Protein

Crude protein content in jackfruit seed powder was determined by the semi-micro-Kjeldahl method with slight modification (Zhang et al., 2008 and Ali et al., 2009). A nitrogen conversion factor of 6.25 was used to compute the protein value and the crude protein content was expressed as a percentage of the total seed content.

3.4.4 Determination of Crude Fat

Moisture free samples were weighed in moisture free thimbles and crude fat was extracted by refluxing in soxhlet apparatus using petroleum ether as a solvent. The percentage of crude fat was calculated by the difference in weight before and after the extraction (Airani, 2007).

$$\text{Crude fat \%} = \frac{Initial\ weight\ (g) - Weight\ after\ extraction\ (g)}{Sample\ weight\ (g)}\ x\ 100 \qquad (3.3)$$

3.4.5 Determination of Crude Fiber

Fat free seed sample was hydrolyzed with dilute sulphuric acid (0.255N) and dilute with alkali NaOH (0.313 N) to estimate crude fibre content by employing the methods of Mayanard (Airani, 2007), as calculated by the following formula:

$$\text{Crude fibre \%} = \frac{Weight\ residue\ with\ crucible\ (g) - Weight\ of\ ash\ with\ crucible(g)}{Weight\ of\ fat\ free\ sample\ (g)}\ x\ 100 \qquad (3.4)$$

3.4.6 Determination of Carbohydrate

The carbohydrate content of the seed was calculated by subtracting the sum of the values for moisture, crude protein, crude fat, crude fiber and ash from 100 % of the total dry matter (Airani, 2007 and Ajayi, 2008).

Carbohydrate = [100 - (crude protein + crude fat + crude fiber + ash)] (3.5)

3.4.7 Determination of Energy

The caloric value was computed by summing up the values obtained by multiplying the values of carbohydrate, crude protein, crude fat and crude fiber with the factors 4, 4, 9 and 2, respectively, and expressed as K calories per 100 g of JSP. The calorific value of the powder was also determined by using a Bomb Calorimeter (Airani, 2007).

3.4.8 Determination of Mineral Compositions

The proximate mineral composition of JSP was determined following the method used by Ajayi, (2008). 1 g of JSP was dried into ash in a muffle furnace at 550 ^{o}C for 5 hr until a white ash was obtained. The minerals were extracted from ash by adding 3 mL of concentrated HNO_3. The digest was carefully filtered into 100 mL standard bottle and made up to mark with distilled water. Minerals were estimated with the use of an atomic absorption spectrophotometer (Perkin Elmer model 703, USA). The instrument was calibrated with standard solutions containing known amounts of the minerals being determined, using analytical reagents.

3.5 ANALYTICAL METHODS

3.5.1 Determination of Protein by Lowry Method

The Lowry method was used to determine crude protein, presented by a color change of the sample solution in proportion to protein concentration, which was measured using ultraviolet spectrophotometry (UV Vis). Preparation of the Lowry assay is explained in detail in Appendix C.

3.5.2 Estimation of Surface Morphology

The scanning electron microscopy (SEM) technique was used to observe the surface physical morphology of the samples by scanning them with a high energy beam of electrons in a raster scan pattern. The samples were closely analyzed by SEM model Leica EM SCD005 and generated high resolution images of shapes and objects. A small amount of sample was coated under argon atmosphere with gold prior to analysis and examined under SEM at 30 kV at range magnifications between 100 to 5000 times.

3.5.3 Estimation of Group Compound

Fourier transform infrared spectroscopy (FTIR) was used to identify the molecular groups of JSP, crude protein, extracted jacalin and standard jacalin. The samples were lyophilized and compressed by KBr method. The grinded KBr powder was pressed with Thermo Nicolet hand press to form a homogenized pellet for background measurement of FTIR spectra, and this was recorded using Varian 660 FTIR spectrometer in the 4000-700 cm^{-1} region (resolution of 4 cm^{-1}) and 16 scans per spectrum in transmittance mode. Sample measurement was taken in the per cent absorbance mode (Simonoska et al., 2013 and Marangoni et al., 2013).

3.5.4 Estimation of Molecular Weight

The molecular weight (MW) of the crude protein and extracted jacalin was determined by SDS-PAGE, comparing to standard jacalin. 3 µL of the sample was separated by SDS-PAGE according to the method of Laemmli (Guzman et al., 2004). Analysis with 15 % resolving and 5 % stacking gels was conducted at 200 V for 55 min using Nu PAGE kits by Invitrogen. The staining gel was fixed, stained and destained according to the manufacturer's instructions for Brilliant Blue G-colloidal concentrate and MW was estimated by comparison with MW markers from BioRad, Hercules, USA.

3.5.5 Protein Sequence Analysis

The isolated fractions were subjected to Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry using ABISCIEX 5800 (Absciex, USA). Protein sequence analyses were carried out using software of ABSCIEX protein pilot. Mass spectra were calibrated externally unless otherwise stated. The samples were analyzed using the MASCOT search engine at www.matrixscience.com. The peptide and fragment mass tolerance were set at 100 ppm and 0.2 Da for MS and MS/MS, respectively (Iwai et al., 2002 and Peng et al., 2009).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 INTRODUCTION

The present research was carried out to study the recovery of crude protein from jackfruit seed powder (JSP) and extraction of jacalin from crude protein. In order to provide a clear understanding of the research work, the results and discussion of this work were arranged accordingly and explained in detail in this chapter. The first part of the chapter explains JSP compositions, followed by the second part of the chapter which focuses on the effect of soaking pretreatment time of the JSP sample prior to performing the recovery process. The optimum soaking pretreatment time which gave the highest result in concentration of crude protein during the recovery process was used as pretreatment time for the whole study. The next part of the discussion focuses on the recovery of crude protein, in which the preliminary study of parameter range used One-Factor-At-Time (OFAT) whereas the optimization of recovery study used response surface methodology (RSM) by Design of Expert (DOE) software. The recovery process conditions that gave the optimum result for crude protein concentration were selected to perform the extraction of jacalin. This part in the chapter discusses in detail the extraction findings under the influence of their respective variables. Then, the last part of the discussion focuses on cytotoxicty performances of jacalin applied to cancer cells (human breast cancer and non-small lung carcinoma) by *in vitro* study using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In order to ensure the reliability of the data, each experiment was conducted at a minimum of 3 times duplication, with a degree of acceptable error within +/- 10%.

4.2 COMPOSITIONS OF JACKFRUIT SEED POWDER

The first part of this study will discuss the composition of the raw material, JSP *Madu Mastura*. The present results show the moisture, ash, crude protein, crude oil/fat, crude fiber and carbohydrate contents of JSP *Madu Mastura* to be 10.10 %, 3.20 %, 12.20 %, 0.00 %, 3.10 % and 74.50 %, respectively. Table 4.1 below shows JSP compositions in comparison of the current study (in bold font) with the previous reported by Tulyathan et al. (2002), Airani (2007), Ajayi (2008), Zabidi and Aziz (2009), Baliga et al., (2011) and Ocloo et al., (2010). The outcome indicates that the major components of JSP are moisture and carbohydrates, 72.47 % and 30.44 %, respectively and 12.20 % of protein. The comparison shows that the crude protein for JSP of *Madu Mastura* is third highest, with 12.20 % followed by 13.50 % and 20.19 % of crude protein by Ocloo et al., (2010) and Ajayi (2007), respectively. The difference in percentage of compositions is due to various factors, among them maturation of the seeds and environmental conditions (Babitha et al., 2007). Bobbio et al (1978) reported that the protein content was very high in seed and starch. However, composition of jackfruit seed of current study is in the range compared to previous reported.

Table 4.1: Proximate composition and physicochemical properties of jackfruit seed powder

Determination	% dry weight in 100g sample						
	Current study	**Tulyathan et al. (2002)**	**Airani (2007)**	**Ajayi (2008)**	**Zabidi and Aziz (2009)**	**Baliga et al., (2011)**	**Ocloo et al., (2010)**
Moisture (g)	**10.10**	7.70	14.07	2.78	5.93	57.75	6.09
Ash (g)	**3.20**	3.97	3.01	6.72	2.21	Not mention	2.70
Crude protein (g)	**12.20**	11.02	9.03	20.19	8.78	6.82	13.50
Crude oil/fat (g)	**0.00**	1.01	1.10	11.39	0.96	0.42	1.27
Crude fiber (g)	**3.10**	2.36	2.55	7.10	2.35	1.25	3.19
Carbohydrate (g)	**74.50**	81.64	70.26	51.82	79.74	32.10	79.34
Energy (kcal)	**347.00**	Not mention	376.00	Not mention	Not mention	136.00	382.79

Table 4.2: Proximate mineral composition of jackfruit seed powder

Determination	% dry weight in 100g sample			
	This study	**Ajayi (2008)**	**Baliga et al., (2011)**	**Ocloo et al., (2010)**
Calcium (mg)	**59.40**	190.00	50.00	308.70
Sodium (mg)	**14.80**	398.50	63.20	6.07
Magnesium (mg)	**119.00**	240.00	54.00	338.00
Potassium (mg)	**1469.60**	2470.00	246.00	1478.10
Iron (mg)	**2.10**	148.50	1.50	13.07
Copper (mg)	**1.10**	22.00	Not mention	1.05
Zinc (mg)	**1.50**	40.85	Not mention	<0.01

4.3 SOAKING PRETREATMENT

It is important to determine the pretreatment step for liquid recovery from solid sample in order to enhance the process and produce high yield in the shortest possible duration. Pretreatment was identified as one of the main factors influencing crude protein recovery. It enhances the recovery rate by increasing crude protein diffusivity in the cellulose structure through an increase in the water phase, or even in some instances breaking cell walls prior to crude protein recovery. It allows the proteins trapped in the cell walls of the seed to be released easier since there is less resistance to mass transfer present.

In this study, pretreatment time was evaluated and the optimum time of soaking as pretreatment prior to performing the recovery process of JSP was determined. Variation of pretreatment time in the range of 0 to 24 hr was conducted to study its effect on the concentration of crude protein through conventional extraction methods that employed PBS, precipitation and filtration. Figure 4.1 shows the effects of varying the pretreatment time on the concentration of crude protein. As can be seen, the concentration of crude protein increased in response to the increase of pretreatment time until 6 hr. The increment of pretreatment time increased the exposure time of the sample, thus softening it so that it expands as the solution enters it, hence promotes the breakage of cell walls. This, in turn, increased the release of protein from the soaked sample. However, under such conditions the cell may burst. In general, plant cells are

protected from bursting by the rigid cell wall that surrounds the cell membrane. As liquid enters the cell, it expands until it pushes up tightly against the cell wall, which pushes back with an equal pressure, so no more water can enter.

Within the range studied, results show increasing crude protein concentration starting without pretreatment sample with the lowest concentration of 22.16 mg/mL. The concentration was 25.62 mg/mL and 26.52 mg/mL at 1 and 3 hr of pretreatment, respectively, then maximum recovery of crude protein concentration was obtained at 6 hrs, which is 29.84 mg/mL. The results indicate a 31.92 % increase in crude protein recovery when the pretreatment was extended to 6 hr. Immediately after 6 hr of pretreatment, the graph trend shows a decrease in the recovery of crude protein concentration up until 12 hr, at 22.38 mg/mL, and retains a similar amount at 24 hr with 22.33 mg/mL. The graph indicates that the decreasing concentration of crude protein recovered reached a plateau at 12 to 24 hr. At this condition, the cell walls of the sample had totally ruptured, and the protein was diluted with liquid, causing a decrease in the concentration of crude protein recovered. Therefore, 6 hr soaking pretreatment time was chosen as the optimum time because samples soaked for more than 6 hr will gave lower concentrations of crude protein due to dilution with liquid (PBS).

Previous study by Kabir (1995) was recovery the crude protein from jackfruit seed without pretreatment prior 24 hr stirring in recovery process and the result gave 8 mg/mL of crude protein concentration. While, Roy et. al. (2005) was soaking for 24 hr prior recovery process with stirred for 3 hr was gave higher result with 88 mg/mL of crude protein concentration. Similar finding of current study, which is crude protein recovery without pretreatment was only 22.16 mg/mL and gave higher concentration with pretreatment for 1 hr, 3 hr and 6 hr was 25.62, 26.52 and 29.24 mg/mL, respectively (Figure 4.1).

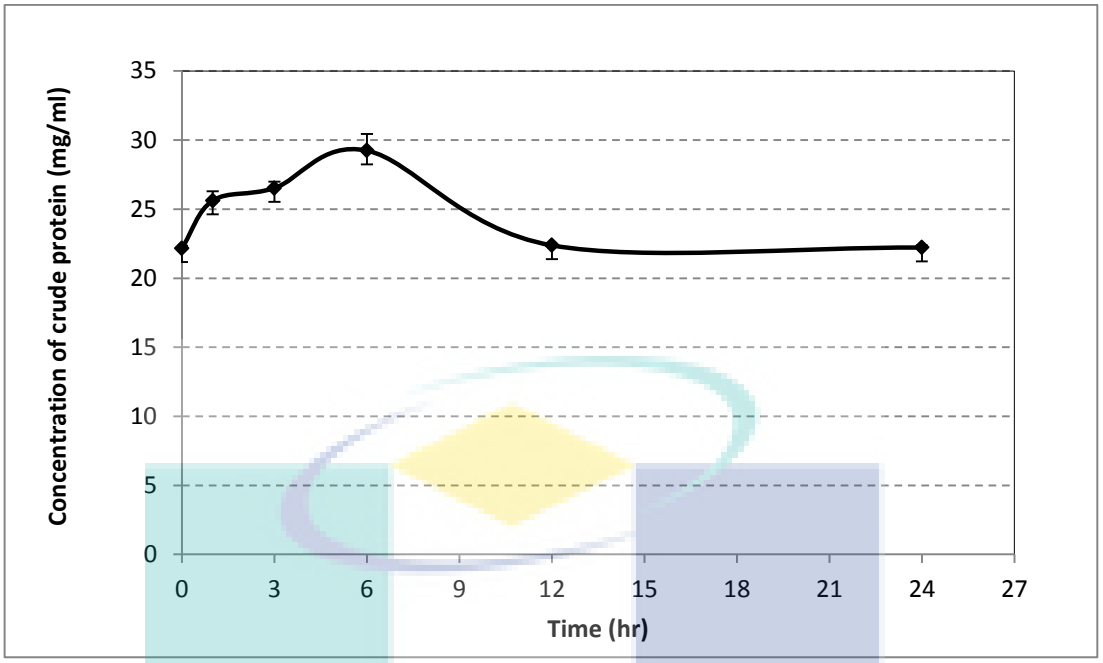


Figure 4.1: Soaking pretreatment time on concentration of crude protein from jackfruit seed powder. The experiments conducted at constant temperature 4^{o}C, liquid to solid ratio 30 mL/g and 5 hr recovery time.

4.3.1 Morphology Effect to Structure of Jackfruit Seed Powder

Scanning Electron Microscope (SEM) was used to visually examine the effects of pretreatment which caused an increase in crude protein concentration recovery. The SEM images show the surface morphology of jackfruit seed powder (JSP) under different conditions of pretreatment time (0, 3, 6 and 12 hr). Different pretreatment times gave different conditions of JSP cell wall surface morphology. The plant cell wall consists of cellulose, hemicellulose, lignin, and pectin, and the wall allows mass transfer through the plasmodesmata. Plasmodesma are microscopic channels which traverse the cell walls of plant cells and some algal cells, enabling transport and communication between them (Nikiforidis et al., 2014).

Figure 4.2 shows the photo of an untreated (without pretreatment) JSP sample. At 500× magnification, when no pretreatment was applied, the picture shows cellulose structures of the cell wall. In comparison, Figure 4.3 shows the ruptured thin-wall cell structure after 3 hr of exposure to soaking pretreatment. It can be seen clearly, at 500× magnification, that the cellulose of the cell wall structure has expanded, and in some instances the cell walls have ruptured. Prolonged pretreatment time increases the cell wall rupturing process. This scenario was observed with samples pretreated for 6 and 12 hr pretreatment, as shown in Figure 4.4 and Figure 4.5, respectively. The cell wall rupture was greater compared to 3 hr of soaking (Figure 4.3)

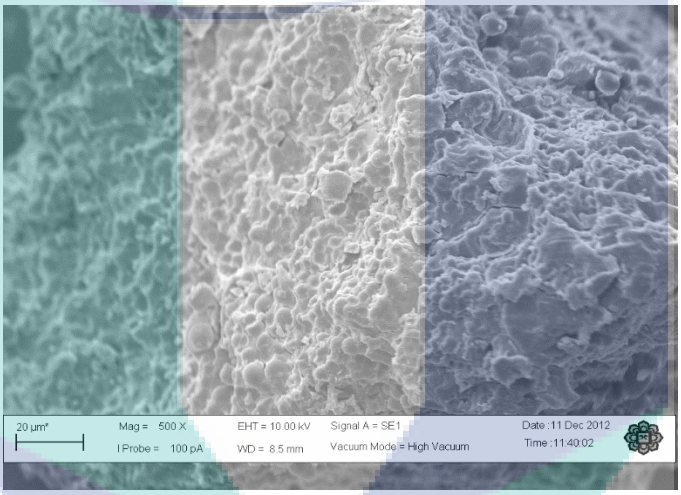


Figure 4.2: SEM photo of untreated (without pretreatment) JSP cellulose

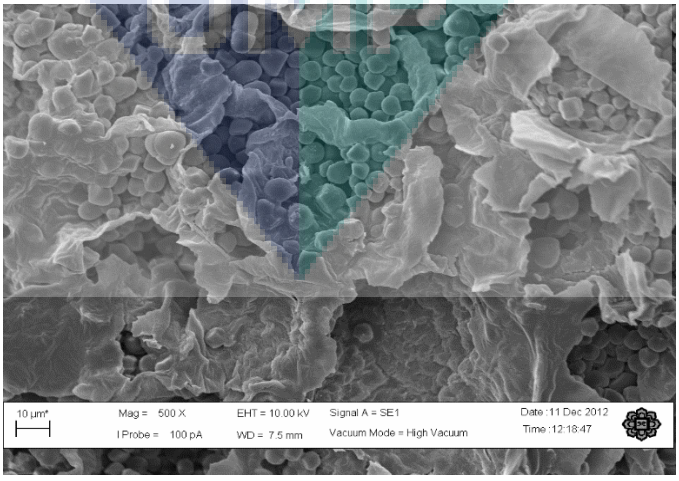


Figure 4.3: SEM photo of JSP cellulose structure after 3 hr of pretreatment

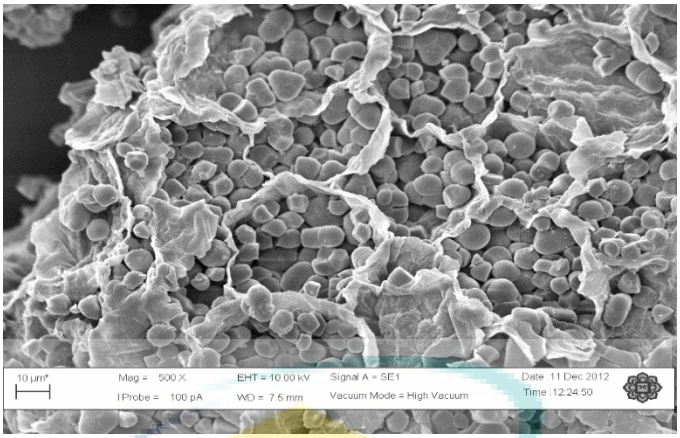


Figure 4.4: SEM photo of JSP cellulose structure after 6 hr of pretreatment

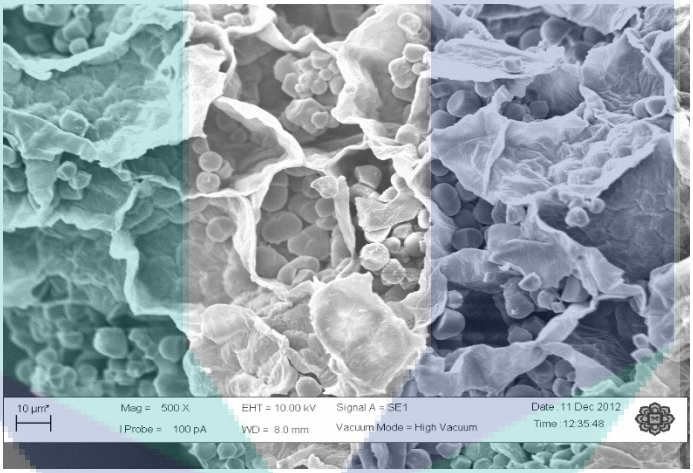


Figure 4.5: SEM photo of JSP cellulose structure after 12 hr of pretreatment

The swelling of the cellulose structure is due to water molecules being absorbed during pretreatment, the exposed solid structure was contacted to water. The increase in water phase within the solid structure assists the transport of protein from the cells to the surface of the solid as a result of concentration driving force at the solid-liquid interface during protein recovery activity. The mobility of protein molecules in liquid phase is far greater than in solid phase. Hence, it is evident that the protein is easily released from the treated sample as a result of cell rupture and diffusion through cell walls in the presence of continuous water phase filling the cellulose structure.

The solute is able to diffuse especially through liquid phase. Compared to the other samples, the untreated sample (without pretreatment) had the least amount of water molecules present in the solid structure due to no time exposure of solid to water prior to the recovery process, making it difficult to extract the protein compound effectively from the solid structure due to the reasons highlighted above. The sample was not effectively wetted, thus water molecules did not thoroughly penetrate the cellulose cell structures. Hence, less protein was able to diffuse through. Exposure of the solid samples to liquid (solution) loosens the tight arrangement of cellulose structure, allowing the penetration of water molecules into the cellulose structure.

A minor experiment was conducted to measure the amount of water molecules in the cellulose structure as a result of pretreating the JSP sample. Figure 4.6 compares three weights of JSP samples: untreated (without pretreatment), 3, 6 and 12 hr soaked. As predicted, the 3 hr soaked sample showed greater weight (58.89± g) than the untreated (pretreatment time equal to 0 hr) sample (10 g). The increase in water content effectively facilitates the release of protein from the solid structure, as reflected in the improvement of crude protein concentration recorded above. Meanwhile, the weight of the sample was even greater after 6 and 12 hr of soaking, at 65.72± g and 81.34± g, respectively. A further 84.78 % weight gain was recorded between untreated (pretreatment time equal to 0 hr) and 6 hr pretreatment processes. By contrast, there was a 31.92 % gain in crude protein concentration (Figure 4.1) between untreated (pretreatment time equal to 0 hr) and 6 hr of soaking pretreatment. This shows that apart from enlargement of the cellulose structure, soaking also, to a certain extent, effectively ruptures the cell walls. This explains the reason for obtaining higher crude protein concentration from the recovery activity. The enlargement of the pores allows more water molecules to be absorbed by the solid structure, hence increasing the water phase and stopping the transfer of protein. This results in a larger opening for water molecules to penetrate the solid structure and thus increase the water phase (Zuraidah, 2010). Pretreatment application prior to recovery activity gives a bigger impact in terms of higher concentration of crude protein recovered. Based on the optimum crude protein concentration as discussed above, 6 hr soaking was selected as the best pretreatment time.

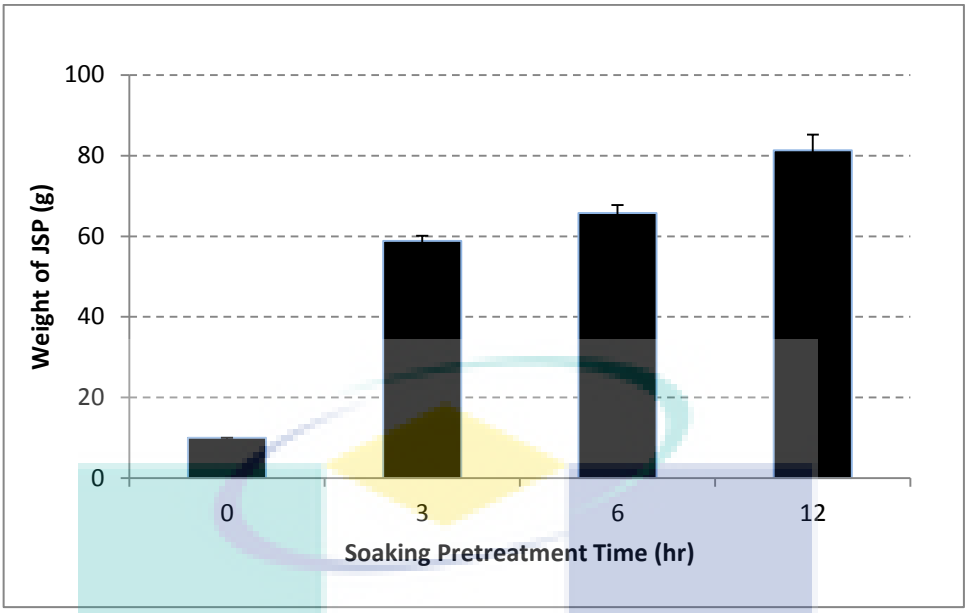


Figure 4.6: Weight of jackfruit seed powder samples after soaking for 0 to 12 hr.

4.4 BATCH PROCESS OF CRUDE PROTEIN RECOVERY

In this section, the results of crude protein recovery from jackfruit seed powder (JSP) are discussed in detail, covering the outcome of varying the variables affecting the recovery process. Three variables, namely the liquid to solid ratio (LSR), recovery time and temperature, were examined to determine the optimum operating condition that gives the highest concentration within the range of the study. Process parameters were screened using the one-factor-at-time (OFAT) approach to select optimum experimental regions. Statistically designed experiments that vary several factors simultaneously are more efficient when studying two or more factors aiming for optimization (Abdul-Hafez et al., 2014). The studies were performed as eight experiments, as suggested by DOE software, within the range of studies for each variable against only one response, which is crude protein concentration.

The number of factors was statistically studied using One Factorial Design by Design of Expect (DOE) software and the statistical analysis of the model was carried out using Fisher's statistical test for the analysis of variance (ANOVA). The input and output variables were fitted to the equation. The fitness of the model was verified by

different criteria, such as *F*-values, *P*-values and determination coefficient (R^2). The *F*- and *P*-values are used as tools to check the significance of each model term coefficient and the interaction strength of parameters. The larger the *F*-value and the smaller the corresponding *P*-value, the more significant is the corresponding coefficient or model term (Montgomery, 2006). The value of R^2 is a measure of the total variation of the observed values of crude protein concentration about the mean as explained by the fitted model, which is often described in percentage. As stated by Montgomery (2006), the closer the R^2 value is to 1.0, the stronger the model and the better the response predictions.

4.4.1 Influence of Liquid to Solid Ratio on Crude Protein Concentration

Numerous levels of liquid to solid ratio (LSR) were investigated to determine the optimum ratio for maximum recovery. Table 4.3 shows the effects of different LSRs (3, 6, 9, 12 and 15 mL/g) on crude protein concentration at constant values of recovery time (3 hr) and temperature (4 ^{o}C). The results illustrate significantly different crude protein concentrations for each different ratio. The highest obtained crude protein concentration was 29.03 mg/mL at the lowest ratio of 3 mL/g (30 mL PBS with 10 g of JSP), and the lowest crude protein concentration was at the highest ratio of 15 mL/g, which obtained only 5.18 mg/mL concentration. Crude protein concentration dropped significantly with further increase of LSR, indicating that the reduced crude protein concentration is likely to have resulted from the lowest solid JSP content. The 10 g of JSP had been suspended in 30 mL amount of PBS with a low liquid ratio, 3 mL/g (high concentration), compared with high liquid ratio at 15 mL/g (low concentration of sample). Crude protein concentration is improved as the quantity of liquid medium used to recover a solid sample is decreased. If more liquid is used for the recovery process, the crude protein concentration in the system is lower and the quantity of protein associated with the retained portion of extract is smaller.

Studies on the effect of LSR in the recovery of jackfruit seed protein could not be traced, however similar studies using other plant materials have been reported. Firatligil-durmus and Evranuz, (2010) worked on protein recovery from red pepper seed powder and found significant effects of LSR, with low yield recovered at high liquid volume. Quanhong and Caili, (2005) studied the extraction of pumpkin seed protein and concluded that maximum yield was obtained by extracting seeds at ratio of 30.2 mL/g and reaction time of 18 minutes. Wani et al., (2008) worked on protein extraction from watermelon seeds and found that optimum extraction could be achieved by extracting seed meal with LSR of 70 mL/g with mixing time of 15 minutes.

Table 4.3: Experimental design and result (actual and predicted value) of liquid to solid ratio effect on crude protein concentration

Run Order	Standard Order	Actual Value, c_a	Predicted Value, c_p	Residual	Student's Residual	Cook's Distance	Outlier t
6	1	29.03	25.38	3.66	1.473	0.577	1.683
3	2	21.48	25.38	-3.89	-1.569	0.655	-1.865
8	3	20.19	20.58	-0.39	-0.139	0.002	-0.127
5	4	13.14	15.78	-2.27	-0.953	0.065	-0.944
1	5	19.99	15.58	4.21	1.466	0.154	1.671
7	6	10.13	10.98	-0.84	-0.304	0.010	-0.279
4	7	5.18	6.18	-1.00	-0.403	0.043	-0.373
2	8	7.17	6.18	0.99	0.400	0.042	0.370

In order to improve recovery of crude protein, LSR was one of the parameters that was studied in order to identify the small range ratio for further optimization study. The linear regression equation from DOE for the liquid to solid ratio, mL/g (*A*), as the main variable, was as follows:

$$\text{Crude protein concentration} = 30.18 - 0.16 * A \quad (4.1)$$

According to Eq. (4.1), the value of estimated regression coefficient for crude protein concentration can be predicted by using liquid to solid ratio, (*A*). Thus, it is worth noting that this equation for crude protein concentration estimated for ratio range

within 3 to 15 mL/g. Table 4.4 summarizes the ANOVA results of one factorial for LSR effect on crude protein concentration. The ANOVA of crude protein concentration demonstrates that the model is significant, as was evident from the high *F*-values (F_{model} = 43.95) and probability value of less than 0.0500 (*Prob* > F = 0.0006). These outcomes ensure a satisfactory fit of the model to the experimental data and implies that the model terms have a significant impact on the response.

Furthermore, the determination coefficient or R^2 was found to be 0.8799, which corresponds to crude protein concentration. This indicates that 87.99 % of the sample variation in the crude protein concentration is explained by the fitted model in Eq. (4.1). The value of R^2 also indicates that only 12.01 % of the total variation was not explained by the model, which is counted as residual. Based on Table 4.4, the Pred R^2 of 0.7754 is in reasonable agreement with the Adj R^2 of 0.8599, the differences are less than 0.2 and it is desirable because a ratio greater than 4 (12.501) is indicative of an adequate signal. According to the data in Table 4.4, this model was significant and can be used to navigate the design space. If there are many insignificant model terms, the model reduction may improve the model. The lack of fit F-value of 0.01 implies that the lack of fit was not significant.

Table 4.4: Analysis of variance for liquid to solid ratio on crude protein concentration

Source		Sum of Squares	DF	Mean Square	F-value	Prob > F	
Model		*414.77*	*1*	*414.77*	*43.95*	*0.0006*	*Significant*
	A	*414.77*	*1*	*414.77*	*43.95*	*0.0006*	
Residual		*56.62*	*6*	*9.44*			
Lack of Fit		*1.98*	*3*	*0.66*	*0.036*	*0.9890*	*Not significant*
Pure Error		*54.65*	*3*	*18.22*			
Cor Total		*471.39*	*7*				
R-Squared							*0.8799*
Adj R-Squared							*0.8599*
Pred R-Squared							*0.7754*

Crude protein concentration from JSP is shown to be significantly decreased when LSR increased from 3 to 15 mL/g. Therefore, LSR 3 mL/g was chosen as the best ratio parameter for further experiments in identifying the overall conditions for optimum crude protein concentration. The main objective of this part was to obtain high crude protein concentration and the amount of PBS used per unit weight of solid can be reduced by performing hydrolysis at lower LSR. Moreover, the lower LSR is more tolerable due to less PBS solution being required. In comparison with previous study of crude protein recovery from jackfruit seed powder by Kabir (1995) was reported 10 mL/g of liquid to solid ratio with 8 mg/mL recovery. While, current study was enhanced with 3 mL/g gave 43 mg/mL crude protein concentration.

Previous study in protein extraction by Stone et al., (2014) reported 3 mL/g of liquid to solid ratio was selected for the pea protein extraction, which is gave higher yield. While, extraction of *sauromatum venosum* lectin was used 5 mL/g with recovery 22% (Bains et al.. 2005). However, Guzman (2004) and Ye et al., (2012) was found 10 and 30 mL/g the optimum liquid to solid ratio for the *vinorama* lectin and beer yeast protein extraction, respectively. Liquid to solid ratio range is different for each sample; it is depending of water solubility of sample. High water solubility of sample requisite more water to dilute the sample.

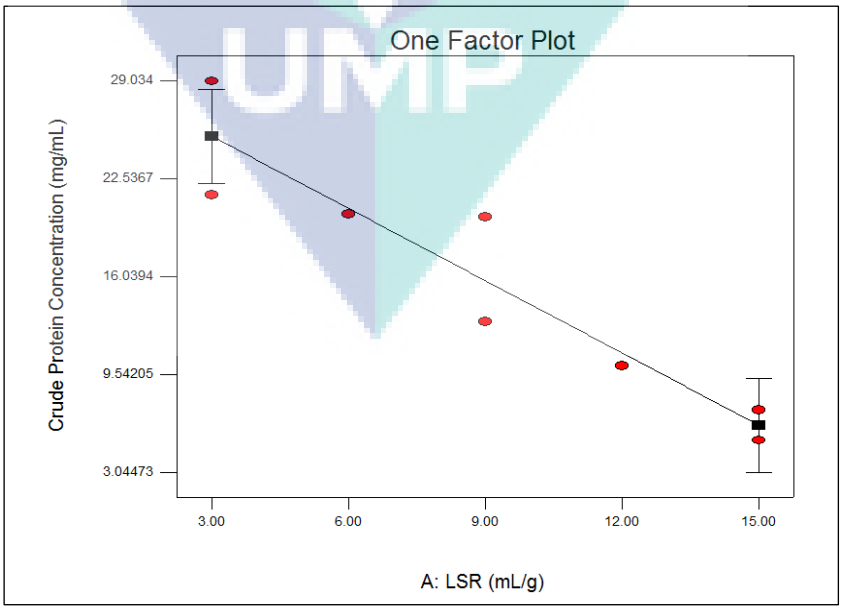


Figure 4.7: Influence of liquid to solid ratio (mL/g) on the crude protein concentration

4.4.2 Influence of Recovery Time on Crude Protein Concentration

Variation of recovery time in the range of 0 to 12 hr (0, 3, 6, 9, 12 hr) was conducted to study its effect on crude protein concentration from JSP at constant values of LSR (6 mL/g) and temperature at 4 ^{o}C. Table 4.5 shows the results of various recovery times on crude protein concentration. As can be seen, crude protein concentration increased in response to increased recovery time. The increment of recovery time increases the exposure time of the sample to recovery activities, hence, increasing the breakage of cell walls. This, in turn, increased the release of crude protein from JSP. Recovery time is crucial for minimizing the energy and cost of the recovery process. Figure 4.8 shows the effect of recovery time on crude protein concentration. Crude protein concentration sharply increased parallel to recovery time from 0 to 6 hr, with concentration 17.06 mg/mL and 33.18 mg/mL, respectively. Conversely, the graph shows the trend decreasing after 6 hr of recovery time, and at 12 hr of recovery time, the graph reached a plateau with crude protein concentration at 26.85 mg/mL.

Table 4.5: Experimental design and effect of recovery time on crude protein concentration

Run Order	Standard Order	Actual Value, c_a	Predicted Value, c_p	Residual	Student's Residual	Cook's Distance	Outlier t
1	1	18.78	17.86	0.92	0.632	0.117	0.589
4	2	17.06	17.86	-0.86	-0.590	0.102	-0.547
2	3	28.32	28.32	-1.20	-0.693	0.051	-0.652
3	4	33.18	38.11	2.07	1.268	0.256	1.377
7	5	33.11	33.11	1.15	0.705	0.079	0.664
5	6	32.38	32.21	-3.10	-1.792	0.341	-2..680
6	7	25.44	25.64	-0.20	-0.135	0.005	-0.121
8	8	26.85	25.64	1.21	0.834	0.203	0.804

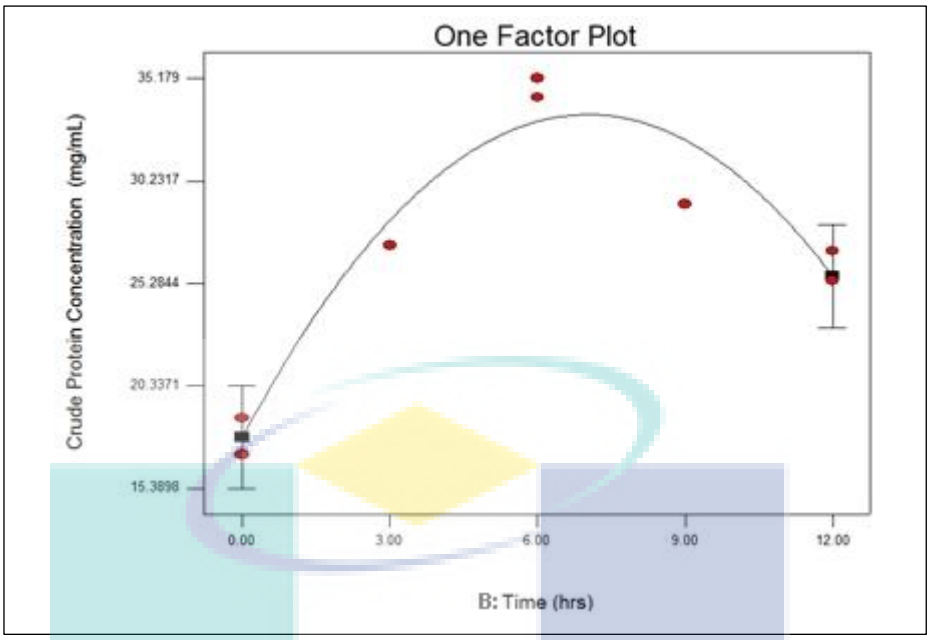


Figure 4.8: Influence of recovery time on crude protein concentration

Two main factors contributed to the increase in the release of crude protein from the JSP sample, namely cell breach and untying of leathery bonding. The first 6 hr of recovery activities play an important role in rupturing the cell walls, which in turn helps release proteins from the solid structure. In the second phase of the slope (between 6 to 12 hr of the recovery process), the crude protein concentration showed a decreasing trend, indicating a decrease in the contribution of proteins released from the solid structure. Hence, it can be concluded that during this first phase, cell rupturing activities are the main contributor to the increase in crude protein concentration. It can be assumed that during the second phase slope, crude protein concentration was reduced because of a slowdown in cell rupturing activities due to most of the cell already having ruptured. On the other hand, it can be suggested that the main contributor to the increase in crude protein concentration is the exposure of solid samples to liquid (solution) loosening the tight arrangement of cellulose structure, allowing the penetration of water molecules into the cellulose structure.

Table 4.6 shows the ANOVA results, calculated by DOE, of the experiments on the effect of recovery time on crude protein concentration, which gives the quadratic regression equation for the recovery time (*B*) as the main variable as follows:

$$\text{Crude Protein Concentration} = 17.86 + 4.43^{*} B - 0.31^{*} B^{2} \quad (4.2)$$

Referring to Eq. (4.2), the value of estimated regression coefficient for crude protein concentration can be predicted using recovery time (*B*). The determination coefficient, or R^2, was found to be 0.9326, which corresponds to crude protein concentration. The value of R^2 is a measure of the total variation of the observed values of crude protein concentration about the mean as explained by the fitted model, which is often described in percentage. This indicates that 93.26 % of the total recovery time variation in the crude protein concentration is explained by the fitted model in Eq. (4.2) and the rest of the total variation was not explained by the model, which is counted as residual. The Pred R^2 of 0.8555 is in reasonable agreement with the Adj R^2 of 0.9057, with the difference at 0.05. The model can be used to navigate the design space with a ratio greater than 4 and it means an adequate signal.

Additionally, the ANOVA of crude protein concentration infers that the model was significant, with *F*-values (F_{model} = 34.60) and probability values too small (*Prob* >*F* = 0.0089). In this case the null hypothesis is rejected if the probability value is less than 0.1000 of significance for crude protein. The lack of fit *F*-value of 8.44 implies that the lack of fit was not significant. These findings confirm a satisfactory adjustment of the model to the experimental data and confirm that the model terms have significant impact on the response.

Moreover, current study was enhanced with 33.18 mg/mL at 6 hr recovery of crude protein from jackfruit seed powder compared to former study by Kabir (1995) which recovered only 8 mg/mL at 24 hr recovery time without pretreatment. This finding showed that soaking pretreatment was reduce in shortens of recovery time for the protein extraction. However, recovery of *sauromatum venosum* lectin required only 15 minutes after overnight of soaking pretreatment while *crataeva tapia* lectin required overnight recovery process (Bains et al., 2005 and Nascimento et al., 2008). Conclude that dissimilar natures of lectin requisite differ optimum recovery time, probably effect of particle size of sample powder and ratio of liquid to solid.

Table 4.6: Analysis of variance for recovery time varied on crude protein concentration

Source		Sum of Squares	DF	Mean Square	*F*-value	*Prob* > F	
Model		*273.58*	*2*	*136.79*	*34.60*	*0.0012*	*Significant*
	B	*68.00*	*1*	*68.00*	*17.20*	*0.0089*	
	B^2	*205.58*	*1*	*205.58*	*51.99*	*0.0008*	
Residual		*19.77*	*5*	*3.95*			
Lack of Fit		*16.78*	*2*	*8.39*	*8.44*	*0.0587*	*Not significant*
Pure Error		*2.98*	*3*	*0.99*			
Cor Total		*293.35*	*7*				
R-Squared							*0.9326*
Adj R-Squared							*0.9057*
Pred R-Squared							*0.8555*

4.4.3 Influence of Temperature on Crude Protein Concentration

The influence of temperature on the crude protein concentration recovered was evaluated and found to vary from 4 to 40 ^{o}C. The experiments were conducted at constant values of LSR (6 mL/g) and recovery time (3 hr). Table 4.7 shows the effects of various temperatures (4, 13, 22, 31 and 40 ^{o}C) on crude protein concentration, based on DOE with predicted and actual data. The results illustrate significantly different crude protein concentrations at each changed temperature. Figure 4.9 presents the effect of temperature on crude protein concentration. Crude protein concentration was increased with rising temperature and reached the highest concentration of 25.81 mg/mL at 31 ^{o}C, and the graph shows the trend decreasing after 31 ^{o}C.

Table 4.7: Experimental design and result of temperature effect on crude protein concentration

Run Order	Standard Order	Actual Value, c_a	Predicted Value, c_p	Residual	Student's Residual	Cook's Distance	Outlier t
7	1	21.29	21.45	-0.16	-0.188	0.010	-0.169
8	2	22.33	21.45	0.88	1.067	0.333	1.085
2	3	22.12	23.86	-1.74	-1.766	0.331	-2.573
3	4	25.30	25.11	0.19	0.204	0.007	0.183
5	5	25.79	25.11	0.68	0.727	0.084	0.688
1	6	25.81	25.22	0.58	0.592	0.037	0.550
4	7	23.06	24.19	-1.12	-1.363	0.543	-1.537
6	8	24.87	24.19	0.69	0.834	0.204	0.804

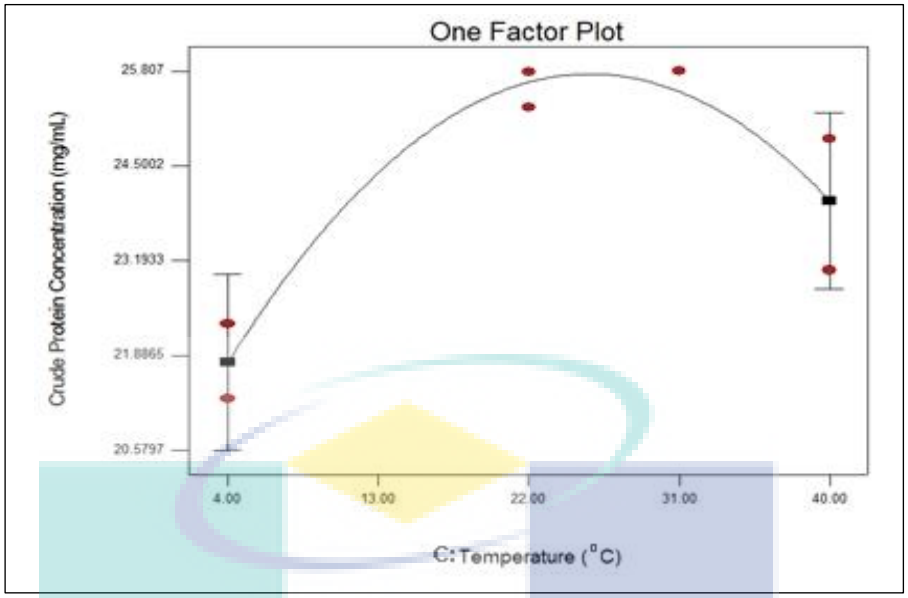


Figure 4.9: Influence of temperature on the crude protein concentration

The quadratic regression equation for the recovery activity of temperature (*C*) as the main variable is shown in Eq. (4.3);

$$\text{Crude Protein Concentration} = 25.19+0.43^{*}\,C-8.47\text{E-}003^{*}\,C^{2} \quad (4.3)$$

The value of estimated regression coefficient for crude protein concentration can be predicted using temperature (*C*), and the ANOVA of crude protein concentration infers that the model was significant with *F*-values (F_{model} = 14.58). The probability values, *Prob>F* (0.001) indicate that the model terms had significant effect on the response of crude protein concentration. Furthermore, the lack of fit F-value of 0.01 implies that the lack of fit was not significant. The coefficient of determination (R^2) was found to be 0.8793, which corresponds to crude protein concentration. The value of R^2 is a measure of the total variation of the observed values of crude protein concentration about the mean as explained by the fitted model, which is often described in percentage. This indicates that 87.93 % of the total temperature variation in the crude protein concentration is explained by the fitted model and only 12.07 % of total variation was not fitted by this model. Table 4.8 shows that the Pred-R^2 of 0.8190 is in reasonable agreement with the Adj-R^2 of 0.5679, and the model is desirable for navigating the design space because a ratio greater than 4 (7.610) indicates an adequate signal.

The results of this study show that the crude protein concentration was linearly increasing with rising temperature, as increased temperature could support the recovery process by increasing both diffusion coefficient and solubility of protein compounds in the liquid (Chew et al., 2011). Besides that, intense heat from liquid was also able to release bounded protein from the cell wall by breaking down of the cell wall's cellular constituents and hence increasing the amount of lectin recovered. Nonetheless, the recovered crude protein concentration started to decrease when the temperature rose beyond 25 ^{o}C. Loss in concentration of crude protein at high recovery temperature was expected due to degradation of the protein molecules which were previously mobilized at low temperature. Thus, the compounds which were recovered under high temperature had lower concentrations as compared to those which were recovered under low temperature.

Table 4.8: Analysis of varians for temperature on crude protein concentration

Source	Sum of Squares	DF	Mean Square	F value	Prob >	
Model	*17.49*	*2*	*8.74*	*14.58*	*0.0146*	*Significant*
C	*5.20*	*1*	*5.20*	*8.67*	*0.0422*	
C^2	*11.11*	*1*	*11.11*	*18.52*	*0.0126*	
Residual	*2.40*	*4*	*0.60*			
Lack of Fit	*0.11*	*1*	*0.11*	*0.14*	*0.7307*	*Not significant*
Pure Error	*2.29*	*3*	*0.76*			
Cor Total	*19.89*	*6*				
R^2						*0.8793*
Adj *R^2*						*0.8190*
Pred *R^2*						0.5679

The effect of temperature on the protein structure of molecular weight by SDS PAGE as shown in Figure 4.10 was tested by varying the recovery temperature between 4 to 40 ^{o}C. As can be seen in Figure 4.10, SDS-PAGE analysis on crude protein recovered at different recovery temperatures showed similar molecular weight bands. This finding means that there were no changes in the protein compounds in terms of molecular weight. Other than that, the molecular weight band between 22 and 31 ^{o}C was clearer and more precise compared to the others. It is possible that at this

temperature, crude protein was recovered at high concentration. Crude protein from jackfruit seed powder was found to be thermally stable and denatured at 45 ^{o}C like many other plant lectins, namely *Artocarpus lakoocha* and *Erythrina indica* (Laija et. al., 2010 and Uraya et. al., 2011). Recovery of crude protein from *mimosa invisa* by Chandrika and Shaila (1987) was conducted at room temperature. However, many study of crude protein recovery was carried out at 4 ^{o}C such as arcelin from tepary bean, *vinorama* lectin from *acacia constricta* and *phaseolus vulgaris* or small black kidney bean (Shade et. al., 1990 ; Guzman et. al., 2014, and He et. al., 2013).

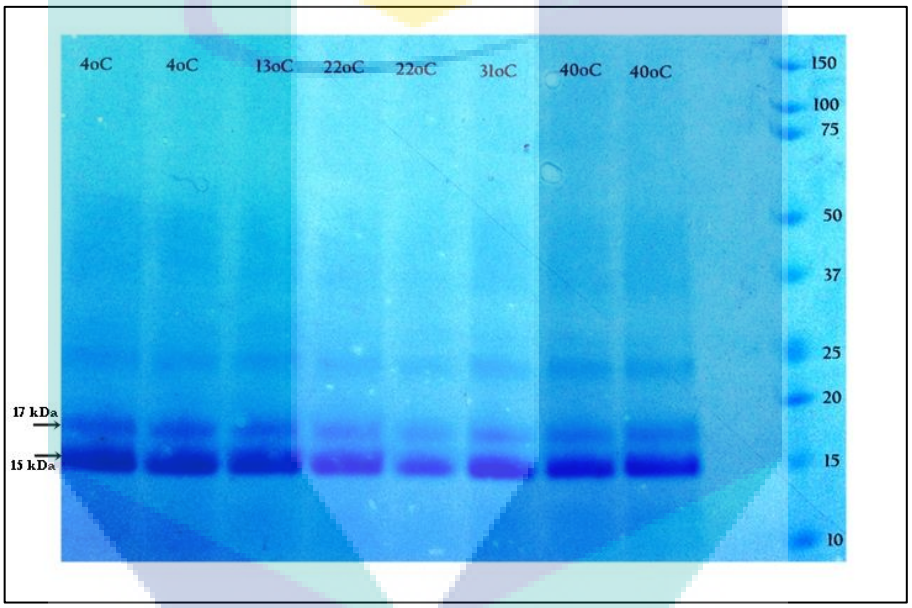


Figure 4.10: SDS-PAGE analysis of crude protein from jackfruit seed powder at different recovery temperatures (4 to 40 ^{o}C).

4.4.4 Summary of Crude Protein Recovery by Batch

The batch process of crude protein recovery experiment in this study was conducted using the One Factor At Time (OFAT) approach based on One Factorial Design by Design of Expert software. The OFAT experiments were a prelude to the experimental design study and optimization, which were included in parameters studies to examine the effectiveness of factors. This OFAT study demonstrated that the recovery of crude protein from JSP was influenced by LSR, recovery time and

temperature alone. The observed optimum levels of LSR, temperature and recovery time were 3 mL/g, 31 ^{o}C and 6 hr, respectively. These conditions resulted in crude protein concentrations of 29.03, 25.81 and 35.18 mg/mL respectively. Therefore, parameter design through OFAT proved to be an important step in optimization studies to obtain the optimum recovery of product without increasing the cost. Thus, the influences of these independent variables were further analyzed by Central Composite Design (CCD) to optimize the recovery process in order to achieve maximum crude protein output.

4.5 OPTIMIZATION OF CRUDE PROTEIN RECOVERY

The design of experiments and the generation of a response surface methodology (RSM) set of experimental runs as well as data analysis were performed using Design of Expert® (DOE) version 6. Afterwards, these factors were optimized using Central Composite Design (CCD) response surface modeling. The significant models showed excellent fitting of the data. Therefore, to support the recovery of crude protein from JSP, the three parameters selected for the CCD were tested. The purpose of optimization in this study is to obtain the highest concentration of crude protein from JSP through the utilization of optimum values of input variables. The RSM is an efficient and popular experimental strategy for determining the optimum conditions for a multivariable system rather than the classical method. Statistical optimization, assisted by DOE software, was performed on the process models and this software offers selection of the chosen goal for each factor and response. The parameters were set to an exact value (factors only) in which a minimum and a maximum level was provided for each parameter included in the optimization. Maximization of desirability, function and recovery level was established using numerical optimization technique in the selected range of parameters. It was based on preliminary work which had identified the optimum combination for LSR, temperature and recovery time, which were set within 25 to 35 mL/g, 25 to 31 ^{o}C and 3 to 5 hr, respectively.

In the present study, the three significant variables, LSR, recovery time and temperature were further optimized using RSM, and experiments were performed according to the experimental design in order to enhance the recovery of crude protein process. The CCD matrix with coded and actual variables for the three independent variables is shown in Table 4.9. A central-composite experimental design with three variables was used to study the response pattern and to determine the optimum combination of variables. The effect of the independent variables *A* (LSR), *B* (recovery time) and *C* (temperature) at three variations levels in the recovery process, is shown in Table 4.10. In this study, a full central composite design with three times replications, three star points and six at the center point, were chosen to allow for estimation of a pure error sum of squares. The design involved 48 runs and the response variable measured was crude protein concentration (mg/mL).

Table 4.9: Factors and levels for the central composite design

Independent Variables	Symbols		Level		
	Uncodified	Codified	-1	0	+1
LSR (mL/g)	X_1	*A*	2.50	3.00	3.50
Recovery time (hrs)	X_2	*B*	3.00	4.00	5.00
Temperature (^oC)	X_3	*C*	25.00	28.00	31.00

Table 4.10: Experimental design and results (actual and predicted value) of the full factorial central composite design

Run	Liquid to solid ratio (mL/g), *A*	Recovery time (hr), *B*	Temperature (^oC), *C*	Crude Protein (mg/mL)		
				Actual, *Yo*	Predicted, *Yi*	*Yo-Yi*
1	25.00	5.00	31.00	42.23	41.48	0.75
2	30.00	6.00	28.00	43.09	44.07	-0.98
3	30.00	4.00	34.00	43.38	45.87	-2.49
4	30.00	4.00	28.00	36.15	39.10	-2.95
5	35.00	5.00	25.00	48.59	45.04	3.55
6	25.00	5.00	25.00	43.09	47.40	-4.31
7	25.00	5.00	31.00	42.88	41.48	1.4
8	35.00	3.00	31.00	58.35	42.61	15.74
9	35.00	3.00	25.00	54.23	39.72	14.51
10	40.00	4.00	28.00	34.78	36.05	-1.27
11	20.00	4.00	28.00	NIL	NIL	NIL
12	35.00	3.00	31.00	27.55	35.96	-8.41
13	30.00	4.00	28.00	40.92	39.10	1.82

Table 4.10: Continued

Run	Liquid to solid ratio (mL/g), *A*	Recovery time (hr), *B*	Temperature (°C), *C*	Crude Protein (mg/mL)		
				Actual, *Yo*	Predicted, *Yi*	*Yo-Yi*
14	30.00	4.00	28.00	38.10	39.10	-1
15	20.00	4.00	28.00	NIL	NIL	NIL
16	40.00	4.00	28.00	34.34	36.05	-1.71
17	30.00	4.00	28.00	39.19	39.10	0.09
18	25.00	5.00	31.00	44.97	41.48	3.49
19	25.00	5.00	25.00	55.53	47.40	8.13
20	35.00	3.00	25.00	26.10	39.72	-13.62
21	35.00	3.00	31.00	34.56	35.96	-1.4
22	30.00	4.00	22.00	47.79	48.91	-1.12
23	30.00	4.00	28.00	39.04	39.10	-0.06
24	35.00	5.00	25.00	53.07	45.04	8.03
25	25.00	5.00	31.00	54.23	46.31	7.92
26	35.00	5.00	25.00	41.72	45.04	-3.32
27	25.00	3.00	25.00	42.73	40.28	2.45
28	30.00	2.00	28.00	41.72	43.58	-1.86
29	25.00	3.00	25.00	35.36	40.28	-4.92
30	30.00	2.00	28.00	43.96	43.58	0.38
31	40.00	4.00	28.00	32.61	36.05	-3.44
32	20.00	4.00	28.00	NIL	NIL	NIL
33	25.00	5.00	25.00	40.42	47.40	-6.98
34	30.00	2.00	28.00	41.14	43.58	-2.44
35	30.00	6.00	28.00	44.61	44.07	0.54
36	25.00	3.00	31.00	41.50	46.31	-4.81
37	30.00	4.00	28.00	34.78	39.10	-4.32
38	35.00	3.00	31.00	41.50	42.61	-1.11
39	30.00	4.00	34.00	44.90	45.87	-0.97
40	30.00	4.00	22.00	47.14	48.91	-1.77
41	35.00	3.00	25.00	39.62	39.72	-0.1
42	25.00	3.00	25.00	48.52	40.28	8.24
43	35.00	5.00	31.00	46.42	35.96	10.46
44	35.00	3.00	31.00	37.52	42.61	-5.09
45	30.00	6.00	28.00	42.01	44.07	-2.06
46	30.00	4.00	34.00	45.55	45.87	-0.32
47	30.00	4.00	22.00	49.17	48.91	0.26
48	25.00	3.00	31.00	41.36	46.31	-4.95

4.5.1 Diagnosis of Model Properties for Recovery of Crude Protein

The statistical properties of a model can best be analysed by inspecting various diagnosis plots such as plot of normal probability, studentized residuals, outlier T, and Box-cox. The most important diagnostic plot is the normal probability plot of the studentized residuals. The studentized residuals are defined as the residuals divided by the calculated standard deviation of those residuals that measure the number of standard deviations separating the actual and predicted values. The normal probability plot (Figure 4.11) depicts a nearly straight line of studentized residuals distribution, which indicates that the errors are evenly distributed, thus supporting the adequacy of the least square fit. The figure also denotes that neither response transformation was needed nor were there any apparent problems with normality.

The plot outlier T is shown in Figure 4.12. The outlier T represents a measure of how many standard deviations of the actual value deviates from the predicted value. Most of the outlier T values should lie in the interval of ± 3.50 and any observation with an outlier T outside of this interval is potentially unusual with respect to its observed response. From the figure it was observed that all the outlier T values fall well within the red lines set at ±3.50, indicating that the approximation of the fitted model to the response surface was fairly good with no data recording error.

In Figure 4.13, the studentized residuals versus predicted response plot is illustrated. Ideally the plot should be a random scatter, suggesting that the variance of actual observation is constant for all values of the response. Figure 4.13 reveals that the proposed model is distinctly adequate and reasonably free from any violation of the independence or constant variance assumption, as studentized residuals lie in the range between 3 and -3. This result also indicates that there is no need for transformation of the response variable (i.e. Box-Cox plot).

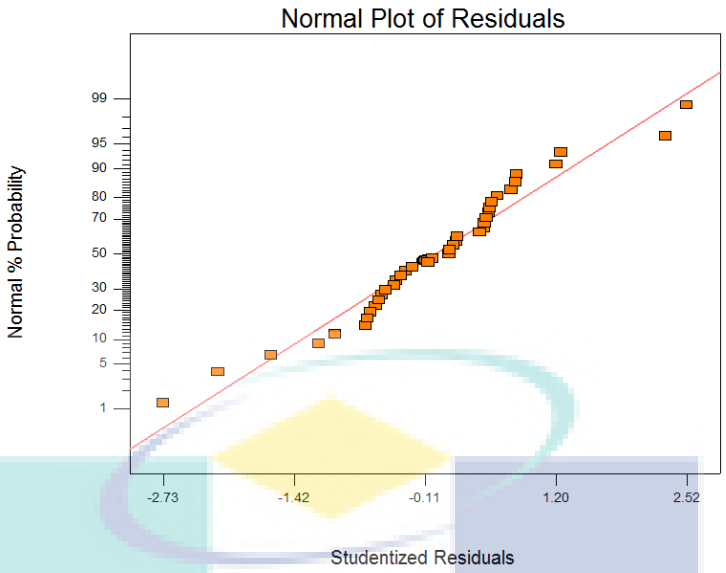


Figure 4.11: Normal probability plots of residuals for crude protein concentration. The residual points follow a straight line which indicates the errors are distributed normally.

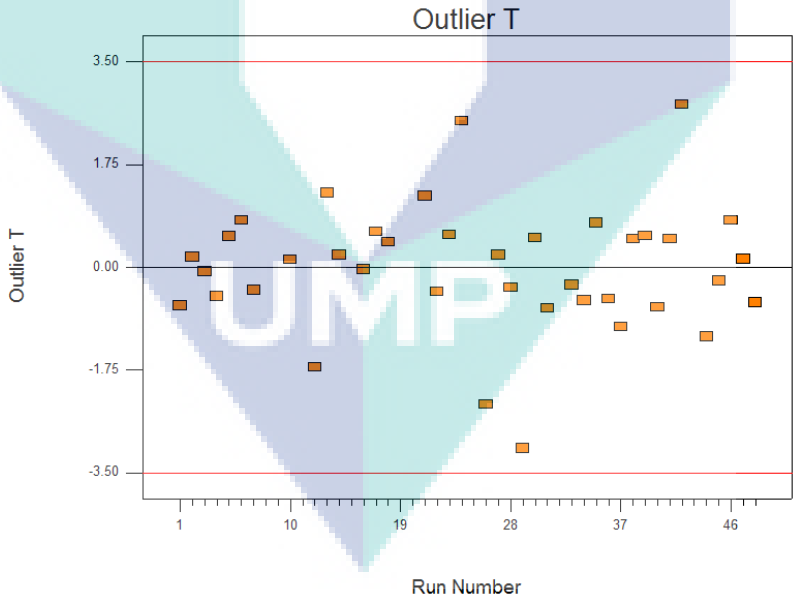


Figure 4.12: The outlier T plot for crude protein concentration

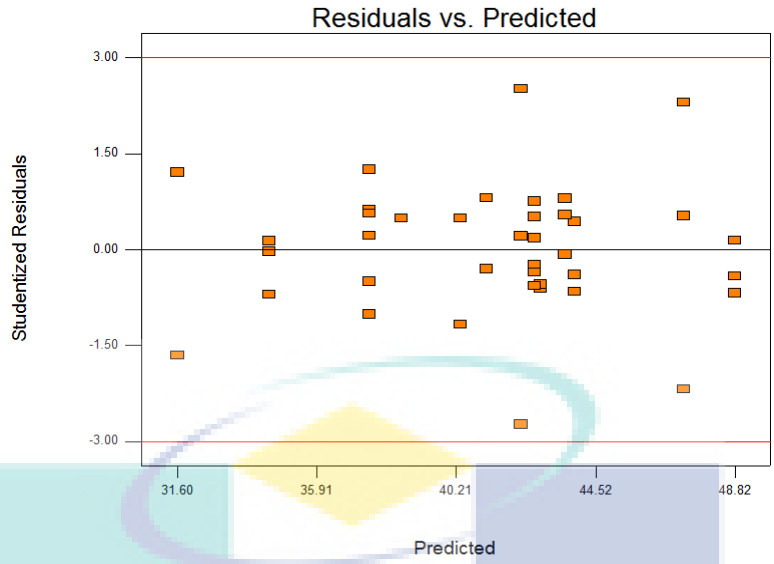


Figure 4.13: The studentized residuals versus predicted response plot is illustrated.

4.5.2 Optimization of Process Condition

A mathematical and statistical approach of response surface methodology (RSM) was applied to solve multivariate equation from quantitative experimental values for recovery of crude protein of jackfruit seed powder. It determines the optimum process parameters in a sequential testing procedure with the advantage of reduced experimental trials, less time consumption, presence of interactions between different variables and efficiency to predict the global optimum (Dutta et al., 2014). The application offers, on the basis of parameter estimate, an empirical relationship between the response variable and the best variables under consideration. In conjunction, by applying multiple regression analysis to the experimental data, the following two-factor interaction (2F1) equation was found to predict as well as optimize the crude protein concentration within the range of variable factors of this experiment. The effects of LSR (*A*), recovery time (*B)* and temperature (*C)* on the recovery of JSP was evaluated. The influence of these input variables was analysed using CCD to determine the optimum conditions that would maximize the concentration of recovered crude protein. The fit summary output analysis denotes that the quadratic models were statistically significant in representing both responses.

The results of ANOVA for the quadratic model representing crude protein recovery are presented in Table 4.14, 4.15 and 4.16 respectively. The model adequacy was checked by *F*-test, *Prob>F* and determination coefficient (R^2). The computed *F* and *Prob>F* values of the model were 13.30 and <0.0001, respectively, indicating that the developed model was highly significant with low probability. Whereas *Prob>F* value being greater than 0.1000 would have indicated that the model terms were not significant. The results adequately suggest that the obtained statistical model was in good prediction of the experimental results and the terms in the model had a significant effect on the response.

Moreover, the R^2 for crude protein concentration recovery was 0.7502, implying that 75.02 % of the variability in the response could be well explained by the model while the remaining 24.98 % of the total variation was elucidated by the residual. The predicted R^2 of 0.5941 is in reasonable agreement with the adjusted R^2 of 0.6938 with a difference of less than 0.2, indicating a good agreement between the observed and predicted data of response. Furthermore, the 'lack of Fit F-value" implies that the lack of fit is not significant relative to the purity of the error. There is a 91.49 % chance that a "Lack of Fit *F*-value" this large could occur due to noise. Each of the actual values, *Yo*, was compared with the predicted value, while *Yi* was calculated from the model, as depicted in Figure 4.14. It can be seen that *Yo* accords with *Yi*.

Table 4.11: ANOVA for the model representing of the crude protein concentration

Source	Sum of Square	DF	Mean Square	*F* value	Prob > F	
Model	753.99	7	107.71	13.30	<0.0001	significant
A	63.77	1	63.77	7.87	0.0006	
C	70.89	1	70.89	8.75	0.0059	
B^2	90.20	1	90.20	11.14	0.0022	
C^2	260.00	1	260.00	32.11	<0.0001	
AC	79.66	1	79.66	9.84	0.0037	
BC	61.75	1	61.75	7.63	0.0096	
ABC	101.44	1	101.44	12.53	0.0013	
Residual	251.04	31	8.10			
Lack of Fit	18.41	6	3.07	0.33	0.9149	Not significant
Pure Error	232.64	25	9.31			
Cor Total	1005.03	38				

Table 4.12: Significance of coefficient estimation, standard error and variance inflation factor for crude protein concentration

Variables	Coefficient Estimation	Standard Error	Variance Inflation Factors
Intercept	37.52	0.85	
A	-1.54	0.55	1.11
C	-1.31	0.44	1.02
B^2	1.28	0.38	1.25
C^2	2.17	0.38	1.25
AC	-2.14	0.68	1.04
BC	-1.92	0.69	1.07
ABC	-2.45	0.69	1.04

Table 4.13: Statistical Analysis

	Response (Crude Protein Concentration)
Standard Deviation	2.85
Mean	41.32
R^2	0.7502
Adjusted R^2	0.6938
Predicted R^2	0.5941
Adequate Precision	13.363

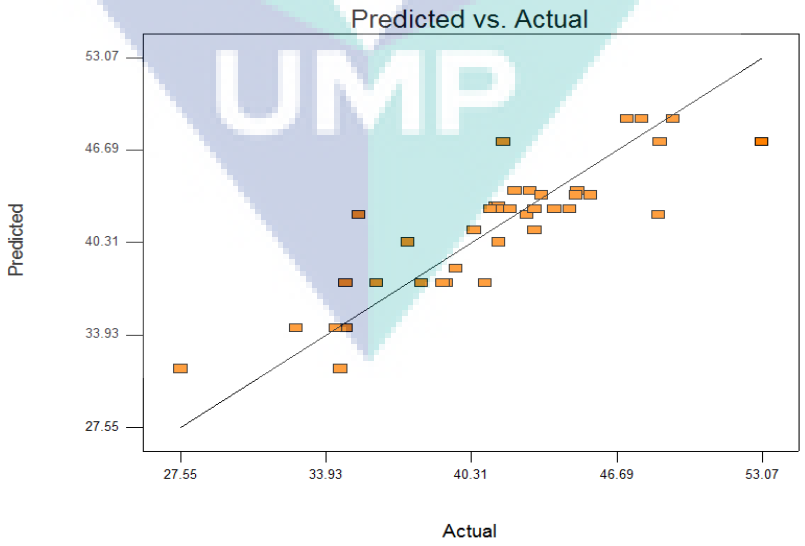


Figure 4.14: Comparison between predicted and actual crude protein concentration

The quadratic effect of temperature (C^2) was found to be the most significant term to have the principal effect towards crude protein concentration, and this was followed by the interaction effect between LSR, recovery time and temperature (*ABC*), the quadratic effect of recovery time (B^2), interaction between LSR and temperature (*AC*), the main effect of temperature (*C*), the main effect of LSR (*A*) and interaction between recovery time and temperature (*BC*). Hence, the ranking of model terms according to their statistical significance (based on the magnitude of *F*-value) in the study of crude protein recovery is as follows: $C^2 > ABC > B2 > AC > C > A > BC$.

The model *F* and *Prob* > *F* values for the model of crude protein concentration were 13.30 and <0.0001, respectively, implying that the model was notably significant with low probability. The R^2 for selectivity was 0.7502, indicating that 75.02 % of the variability in the response was well explained by the model, while only 25.98 % of the total variation was described by the model. In addition, the model showed insignificant lack of fit (*Prob*>*F* = 0.9149) which suggests that the model was desirably fit in representing selectivity. From the table it can be remarked that the difference between the values of adjusted determination coefficient (adj R^2) and predicted determination coefficient (pred R^2) was less than 0.2 with 0.6938 and 0.5941, respectively, denoting a good agreement between the experimental and predicted values of response.

The application of RSM yields the following regression equation, which is an empirical relationship between crude protein concentration and the test variable in coded units, as a function of LSR (*A*), recovery time (*B*) and temperature (*C*), as shown in the following equation:

$$Y = 37.52 - 1.54A - 1.31C + 1.28B^2 + 2.17C^2 - 2.14AC - 1.92BC - 2.45ABC \quad (4.4)$$

where *A*, *B*, and *C* are coded forms of the test variables as described in Table 4.9. The obtained empirical equations are mathematical models that best describe the correlation among the independent variables and the studied responses. Hence, these models can be used to predict and optimize the concentration of recovered crude protein within the experimental constraints.

4.5.3 Interaction Effect Variables

The interaction and response surface graphs were generated to estimate the concentration of crude protein recovered as a function of independent variables A, B, and C, as shown in Figures 4.15 and 4.16. The significance of interactions among the variables on the responses can be better understood using an interaction graph.

Effect of Liquid to Solid Ratio and Temperature on Crude Protein Concentration

The effect of temperature and liquid to solid ratio of crude protein concentration when recovery time was set at 4 hr as a center point is shown in Figure 4.15 (a and b). It is evident that the predicted concentration of the recovered crude protein continuously decreased with the increase in liquid to solid ratio (from 42.06 mg/mL at 25 mL/g to 34.69 mg/mL at 35 mL/g), when temperature was maintained at 25 ^{o}C. Conversely, an opposite result was found at a low level of temperature (31 ^{o}C), in which the crude protein concentration increased (from 40.40 mg/mL at 25 mL/g to 41.60 mg/mL at 35 mL/g) (Figure 4.15 (a and b)). This demonstrates a negative correlation with LSR (mL/g) and a positive correlation with temperature. Besides that, the maximum crude protein concentration was observed at 42.06 mg/mL, corresponding to the point defined by temperature 25 ^{o}C and solid to liquid ratio 10:35 w/v. Moreover, the range of temperature and LSR for the protein recovery activity was parallel with previous processes, such as protein recovery from germinated pumpkin seed, which found significant effects of LSR at 30.2:1 (v/w), and extraction of watermelon seed protein at temperature of 40 ^{o}C (Firatligil and Evranuz, 2010). LSR also plays an important role in affecting the concentration of crude protein yield in the system. Continuous implosions would enlarge the pores and the enlargement of the pores is prudent for more water molecules to be absorbed by the solid structure, hence, increasing the water phase which in turn eases the transfer of oil during extraction.

Effect of Recovery Time and Temperature on Crude Protein Concentration

The influence of recovery time and temperature on recovered crude protein concentration at constant LSR (30 mL/g) is shown in Figure 4.16 (a and b). It is clear that the predicted crude protein concentration reduced with time (from 41.57 at 3 hr to 37.73 at 5 hr of recovery time) at the higher temperature (31 ^{o}C). A reciprocal result was found at low level temperature (25 ^{o}C), in which the crude protein concentration increased linearly from 40.36 to 44.20 at recovery time 3 and 5 hr, respectively (Figure 4.16 (a and b)). This reveals the response surface and interaction plots of crude protein concentration as a function of temperature and recovery time. It is evidently shown that the least favorable crude concentration yield occurred at higher temperature and high recovery time, which is 37.22 mg/mL crude protein concentration yield at temperature of 31 ^{o}C and recovery time of 5 hr. Besides that, the maximum crude concentration was observed at 44.20 mg/mL, corresponding to the point defined by temperature 25 ^{o}C and recovery time 5 hr. On the other hand, recovery time plays an important role in increasing the efficiency of recovery at higher levels (Kumar et al., 2013) to ensure the solid has enough time exposure to liquid for diffusion and transportation of the protein molecules from the solid to liquid.

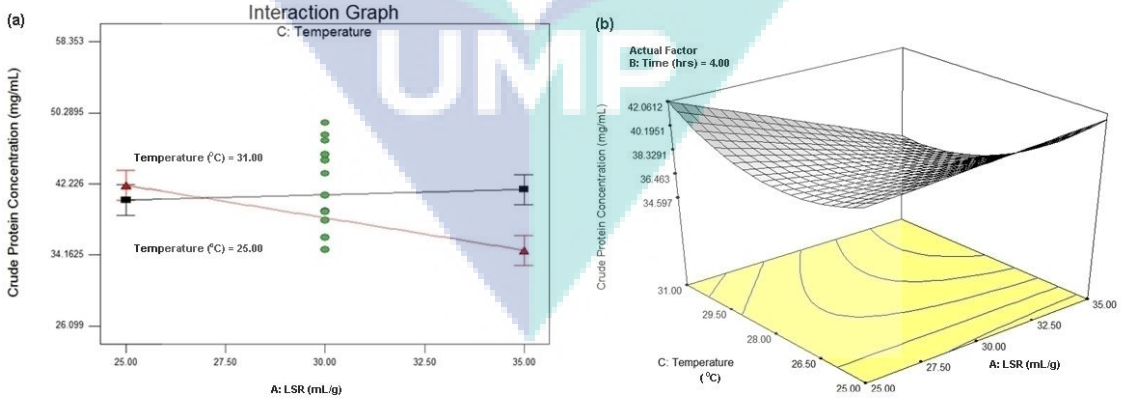


Figure 4.15: Interaction graph (a) and response surface (b) on effect of liquid to solid ratio and temperature on crude protein concentration.

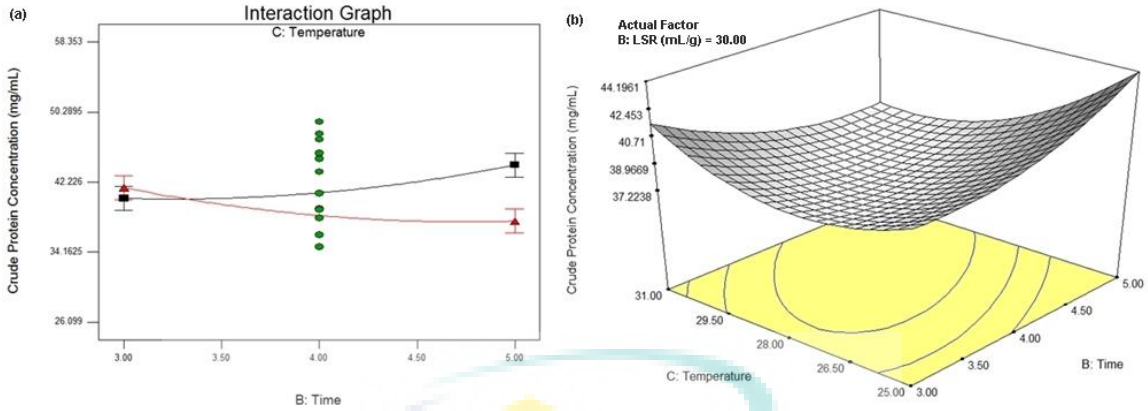


Figure 4.16: Interaction graph (a) and response surface (b) on effect of recovery time and temperature on crude protein concentration.

4.5.4 Validation of Empirical Model Adequacy

Validation of the development empirical model adequacy is necessary to justify prediction accuracy. Based on the models, numerical optimization was executed using the 'Design Expert' program and suggested optimal conditions were obtained, which are shown in Table 4.14 to verify these conditions, batch recovery runs were conducted in triplicate under the recommended optimum conditions. The acquired actual values and their associated predicted values from the verification runs were compared for residual and percentage error analysis. Accordance to Eqs. 4.5, the error in percentage among the actual and predicted values of both responses over a considered range of operating variables were calculated.

$$Residual = (Actual\ Value - Predicted\ Value) \tag{4.5}$$

$$\%\ Error = \frac{Residual}{Actual\ value}\ x\ 100 \tag{4.6}$$

In order to validate the reliability of the models, a series of additional experiments were conducted by varying the three independent variables (Table 4.14) and estimating the crude protein concentration from Eq. 4.4. The first three conditions

of the confirmation run were taken from the optimum experimental conditions which were recommended by the software. The best result for recovered crude protein concentration was obtained at 42.84 mg/mL, with LSR at 30 mL/g, 5 hr of recovery time and temperature at 25 ^{o}C, and this result is typed in boldface in Table 4.14. The results in Table 4.14 also show that the percentage errors from crude protein concentration yield ranged from 7.42 to 8.20 %, hence indicating that the empirical models developed were noticeably accurate for crude protein concentration since the percentage error between the actual and predicted values was agreeable within the value of 10 %, signifying that the model adequacy is reasonable within 90 % of the prediction interval.

Table 4.14: Results of crude protein recovery parameters in validation runs

A **(mL/g)**	***B*** **(hrs)**	***C*** **(^{o}C)**	**Crude Protein**			
			Actual (mg/mL)	**Predicted (mg/mL)**	**Residual**	**Error (%)**
30	**5**	**25**	**42.84**	**44.20**	**-3.18**	**7.42**
30	4	25	40.01	41.23	-3.05	7.62
30	3	30	39.91	41.22	-4.84	8.20

4.5.5 Confirmation Run of the Predicted Optimization Condition

Confirmation testing is an important and necessary step in the response surface method as it is a direct proof of the empirical model obtained. In order to substantiate the predicted optimization conditions, an experiment was carried out using the conditions proposed by optimization mode and the results are demonstrated in Table 4.15. The LSR, temperature and recovery time were set to the values defined previously as the optimum conditions for giving the maximum value of crude protein concentration from the model. The optimum LSR, recovery time and temperature were determined as 30 mL/g, 5 hr and 25 ^{o}C, respectively. To confirm this result, confirmation experiments were performed by employing the model-suggested optimum conditions and the crude protein concentration was 42.84 mg/mL, while the optimum predicted crude protein concentration was 44.20 mg/mL. Apparently, the experimental results show that the optimal value of crude protein concentration was 7.42 % error (Table 4.14), which was found to be very close to the predicted values and thus the model was successfully

validated. Therefore, the obtained model could reliably be utilized for the prediction of optimum crude protein concentration with respect to maximal recovery of crude protein. Furthermore, Table 4.15 also reveals the comparison between results obtained from the experimental condition before and after optimization condition, in which the values after optimization were 29.11 % greater than their values before optimization, hence optimum recovery could be achieved using the response surface method.

Table 4.15: Summary of recovery process using experimnetal design for crude protein from jackfruit seed powder

	Before Optimization	After Optimization
Parameter:		
LSR (mL/g)	6	3
Temperature (^{o}C)	4	25
Recovery time (hr)	6	5
Response:		
Crude protein concentration	33.18	
a) Actual (mg/mL)		42.84
b) Predicted (mg/mL)		44.20

4.6 EXTRACTION OF JACALIN BY REVERSE MICELLE METHOD

The crude protein of jackfruit seed powder (JSP) contains several proteins, including jacalin. However, in a single run lasting only 6 hr, it was possible to isolate pure jacalin fractions containing the characteristic 12 and 15.4 kDa peptides (Kabir, 1995). Liquid-liquid extraction processes using reverse micelles are used in many industrial applications such as enhanced oil recovery and separation schemes such as phase transfer catalysis as well as various types of chemical reactions. It has been validated to be capable of hosting large quantities of biomolecules such as proteins, hemoglobins and plasmids without causing denaturation, despite adjusted operational parameters (Lau et al., 2008). Commonly, protein extraction studies have used sodium bis(2-ethylhexyl) sulfosuccinate (AOT) along with isooctane as the anionic surfactant due to their ease of forming reverse micelles as well as their stability in contrast with other surfactants, and they have been applied in many published studies of other systems with success. AOT is a negative surfactant and thus easily forms reverse micelle with proteins (which are positively charged) (Setapar, 2008).

However, the concentration of jacalin extracted from crude protein is influenced by the distribution of proteins between micelle organic phase and aqueous solution during forward and backward extraction. Hence, the factors influencing protein distribution are the main important subject of study. The distribution of proteins is largely determined by mechanisms based on electrostatic and hydrophobic interactions which depend on the conditions in the aqueous bulk phase, namely pH, ionic strength, type of salt and temperature. The phase transfer depends on the specific characteristics of the protein, namely the isoelectric point, size and shape, hydrophobicity and charge distribution (Melo et al., 2001). The utilisation of reversed micelles in jacalin extraction from crude protein extract was successfully implemented in this study. Two parameters influencing protein distribution are pH and ionic strength (salt concentration). Therefore, the micelle phase concentration of surfactant (AOT) in isooctane was 0.1 M and initial protein concentration was 42.84 mg/mL, which was kept constant in all experiments.

4.6.1 Effect of Ionic Strength on Extraction of Jacalin from Crude Protein

Ionic strength is known as one of the factors that can affect reversed micelles extraction efficiency. It was found earlier that reversed micelles extraction of proteins substantially depends on the salt concentration in the aqueous solution. The organic phase was observed to become cloudy at high salt concentration because the surfactant molecules started to migrate from the isooctane into the aqueous phase (Chuo et al., 2014). The salt concentration, such as NaCl, is the major determining factor on the extent of interaction between the biomolecule and surfactant head group (He et. al., 2013). Figure 4.17 shows the concentrations of jacalin extracted from crude protein at different concentrations of NaCl, from 0.1 to 2.5 M.

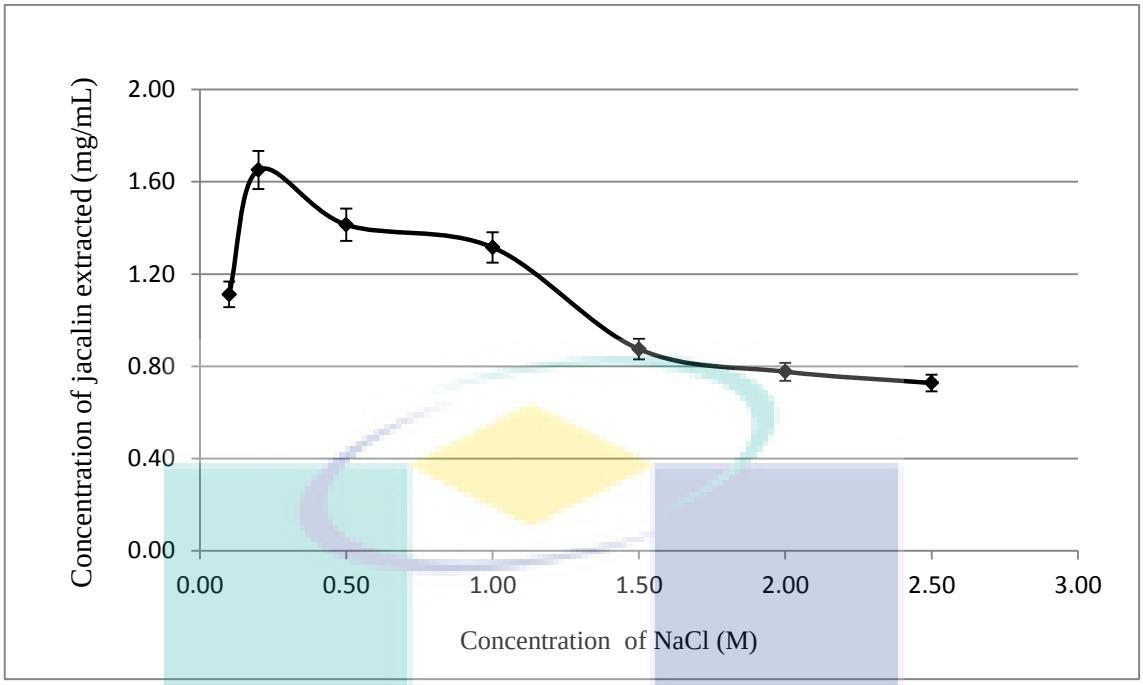


Figure 4.17: The effects of salt (NaCl) concentration on the amount of jacalin extracted into the micellar phases. Forward extraction was carried out using crude protein (pH 6-7), AOT (0.1 M) at room temperature.

From Figure 4.17, it can be seen that the concentration of extracted jacalin increased when NaCl concentration increased up to 0.2 M, with maximum jacalin extracted at 1.65 mg/mL. However, above NaCl concentration 0.2 M, the amount of jacalin solubilized decreased with further increase of salt concentration. Adding low amounts of NaCl helps to stabilize the reversed micelles. However, at the same time, the NaCl reduces the electrostatic interaction between AOT and jacalin. When NaCl becomes too high, less jacalin is captured by the reversed micelles due to lower electrostatic interaction. In addition, NaCl also reduces electrostatic interactions between AOT head groups and leads to smaller reverse micellar size. Thus, the concentration of the jacalin extract was decreased at high NaCl concentration because the reversed micelles became too small for solubilisation of jacalin. This is supported by the findings of Chuo et al., (2014), on the extraction of amoxicillin using mixed reversed micelles system.

Meanwhile, the data presented in Figure 4.17 show that the concentration of jacalin is lower at 0.1 M, which is a similar situation that has been reported for the extraction of lectin from small black kidney beans using reversed micelles system. The solution became cloudy because of very low ionic strength (NaCl/KCl concentration ≤ 0.1 M) (He et al., 2013). Additionally, Chuo et al., (2014) concluded that reversed micelles are not formed without the presence of salt in the system according to the finding that there was no amoxicillin solubilisation detected at salt concentration equal to zero. The concentration and strength of ions is one of the key factors in the reversed micelles system since it can significantly affect the electrostatic interaction between surfactants and solutes in several ways (Dong et al., 2009). The increase in salt concentration up to 0.2 M could maintain the optimum protein transfer, which might be just the sufficient salt concentration to enhance the interaction between jacalin and surfactant head, and reduce the repulsive forces between the two surfactant heads, resulting in the formation of stable reversed micelles. However, when increasing NaCl concentration beyond 0.2 M, the amount of extracted jacalin was found to decrease dramatically with the increase in salt concentration (Figure 4.17).

There are two reasons that can explain this phenomenon. Firstly, a high amount of salt in aqueous solution could reduce the Debye length of protein. Thereby, the electrostatic interaction between protein and surfactants was reduced due to increase in the electrostatic screening effect, resulting in a decrease in protein extraction efficiency. Secondly, as ions control the repulsion force of surfactant head groups, a higher ionic strength could decrease the size of reversed micelles, causing a gradual expulsion of the proteins which is defined as a squeezing-out effect. In the present study, at NaCl concentration 0.2 M, the ionic strength might play a dominant role in excluding the possibility of hydrophobic interactions between the proteins and the surfactant tails. The extraction efficiency increases with an increase in salt concentration until reaching a maximum value at 0.2 M and then decreasing with further increases in salt concentration.

According to Dong et al., (2009) in their study on glutathione extraction using reversed micelles system, the affecting mechanism of ions on reversed micelles extraction can be divided into two parts. Firstly, cations can enhance the dissociation of protein in aqueous phase due to the salting-in effect, thus changing the charge status of proteins and improving the extraction process. Secondly, the cations can form cation–surfactant complexes, which screen the binding of protein with surfactant and decrease the extraction efficiency. At low anion concentration, the salting-in effect dominates while at higher anion concentration, the screen effect prevails instead. Therefore, the experimental results show that the amount of extracted jacalin first increases with the increase in anion concentration until reaching a maximum point, then decreases with further increases in anion concentration. This mechanism is supported by He et al., (2013), as increase in salt concentration up to 0.3 M in their study on lectin extraction from black small bean, resulted in increased extraction efficiency of small hydrophilic protein due to the salting-in effect.

The presence of water structure in the formation of salt (NaCl) enhances the stability of the reversed micelles, causing a higher extent of forward extraction efficiency (Anjana et. al., 2010). The increment in NaCl concentration means a decrease of water content in the system, resulting in small solubilisation of protein into reverse micelles. However, the effects of ionic strength in the solubilisation of proteins in reverse micellar biphasic systems are necessary. There is competition with ionic species for transfer into the reversed micelles and changes in the electrostatic state of the micelles and/or proteins. It has been noted that when the ionic strength of the aqueous phase is below a certain limit, reversed micelles and phase separation do not occur and a stable microemulsion is formed (Nascimento et al., 2008). Therefore, the transfer of proteins between phases requires a minimum value for the ionic strength of the aqueous solution (Melo et al., 2001). The results of this study suggest that the NaCl in the range 0.1 to 0.2 M could maintain the protein transfer at high value. Hence, in the present study, the results suggest that NaCl concentration 0.2 M could maintain the optimum protein transfer and this was maintained in further studies.

4.6.2 Effect of pH on Extraction of Jacalin from Crude Protein

In protein extraction using reversed micelles, the pH of the initial aqueous phase is the major operating parameter that manages the behavior of protein extraction. The pH of the aqueous solution determines the remaining charge of proteins. Electrostatic interactions between proteins and surfactant heads group can favor the transfer of protein into the organic phase. This trend has been observed using anionic and cationic surfactants (Naoe et al., 1998; Melo et al., 2001 and Anjana et al., 2010). Thus, the effect of pH on jacalin extraction was studied whereas salt (NaCl) concentrations were kept constant at 0.2 M. The pH of the first aqueous phase containing the desired protein was varied between pH 5.0 to 12.0 using freshly prepared buffer. The forward extraction of jacalin from crude protein in the pH range studied is shown in Figure 4.18. Each extraction was carried out at a buffer concentration of 0.1 M, with 30 min shaking in an incubator shaker at 400 rpm and centrifugation for 10 min at 3000 rpm. The highest concentration of jacalin extracted was achieved at pH 6.0 with concentration 1.781 mg/mL. At pH 5.0 to 6.0 the extraction concentration sharply increased, then immediately decreased after pH 6.0 (Figure 4.18).

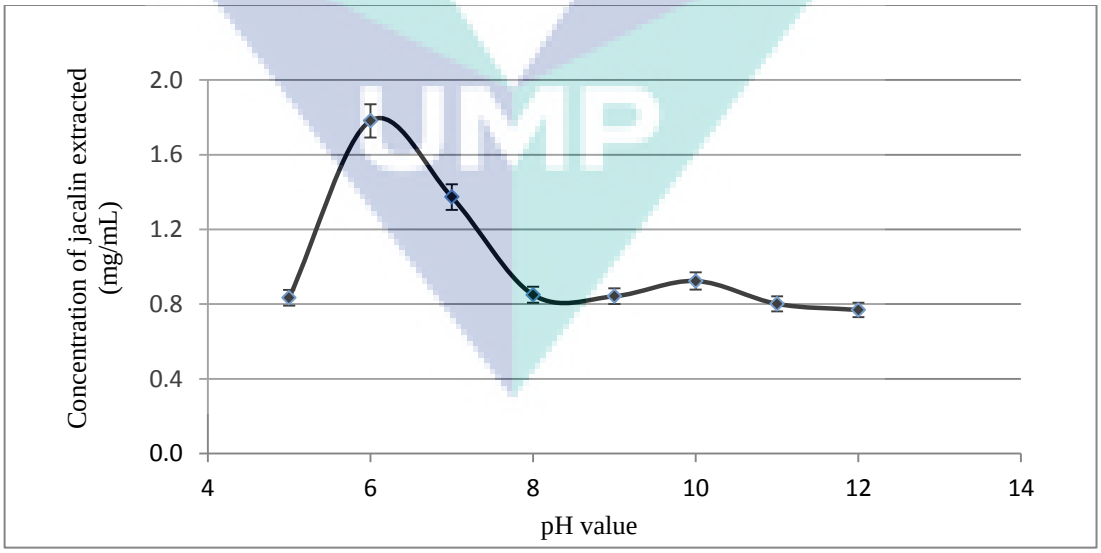


Figure 4.18: The effects of pH on the amount of jacalin extracted into the micellar phases. Forward extraction was carried out using crude protein (0.2 M NaCl), AOT (0.1 M) and kept at room temperature.

The concentration of jacalin extract increased from pH at 5 to 6 with 0.834 and 1.781 mg/mL, respectively (Figure 4.18). However, concentration of jacalin extracts decreasing when the pH of aqueous phase increased above 6.0. Probably at this point the pH value was higher than pI value, which is the pI of jacalin was within 4.9 to 7.1 (Ahmed and Chatterjee, 1989; Sahasrabuddhe et al, 2006). The protein molecules became negatively charged once pH value was higher than pI value and repulsion of interaction with surfactant head (negative charge). Therefore, the concentration of jacalin extract was decreased by 34 % from pH 6 to 8. These results are in line with the finding by Anjana et al., (2010), that degradation of lactoferrin is due to highly basic pH, hence decreasing the amount of protein extracted. The exposed protein hydrophobic patches may interact with cationic surfactant hydrophobic tails, minimizing extraction efficiency. Besides the protein charge, the density of surface charge is an important factor for protein solubilization. However, the degree of extraction also depends on the types of the sample used (Setapar et al., 2012).

Furthermore, in the pH range 8.0 to 12.0, although a stable extract concentration was obtained, a small insoluble precipitate often appeared at the phase boundary. This study suggests that extraction of jacalin in the pH range 5.0 to 6.0 was more stable as insoluble aggregates did not appear. Meanwhile, in the pH range 8.0 to 12.0, jacalin was not extracted into the reverse micelle phase. This finding is similar to previous studies by Naoe et al., (1998), Lau et al., (2008) and He et al., (2013) on extractions of cytochrome and lysozyme, antibiotic penicillin G and small kidney bean, respectively, which found that optimum extraction was at pH 6.0, and extraction was not successful at pH more than 8.0. From this outcome it can be concluded that extraction at pH 6.0 is more selective, probably due to favorable attractive electrostatic interactions between positively charged jacalin molecules at pH 6.0 and negatively charged surfactant head.

Additionally, it can be hypothesized that at lower pH, surfactant (AOT) exists as a monomer of undetached form. It does not adsorb at the interface and hence there is no lowering of interfacial tension to form reversed micelles due to high concentration of hydrogen ions in the aqueous solution. At medium pH range, some AOT molecules detach to form anions, which adsorb at the interface and reduce the interfacial tension. However, the interfacial tension is not low enough to form a clear and

thermodynamically stable reversed micelles solution due to the lack of AOT ions adsorbed at the interface. At higher pH, water solubilisation reaches saturation, indicating that all molecules have detached (exist as anions) to form reversed micelles. Meanwhile, extraction at higher pH can be hypothesized to be a result of hydrophobic interaction between non-polar group surfactant and protein through the formation of hydrogen bonds. The solubility of jacalin drops with increasing pH remaining to the detachment of jacalin. The formation of hydrophobic complex of the protein and AOT molecules makes it possible for proteins to be solubilised into the organic phase since the solubility of the proteins in pure isooctane is zero, even at low pH (Lau et al., 2008).

According to (He et al., 2013), solubilization of protein is favored at pH values below the isoelectric point (pI) of the protein in the case of AOT as anionic surfactant. Hence, higher pH-pI values resulted in increasing the electrostatic interaction between solute molecules and surfactant, which might be a major reason for the increased extraction of hydrophilics from crude protein. Furthermore, the positive charge of the protein molecule surface was opposite in sign to the AOT head group, so there was an attraction between protein surface and the polar head group on the internal surface of the reversed micelles (Melo et al., 2001). However, loss of protein was observed at the phase interface below pH 5.0, which could be explained by protein denaturation. Protein denaturation and changes in the ionisation state of the surfactant at extreme pH values make interpretation of the phase-transfer pattern additionally difficult (Melo et al., 2001). On the other hand, Lau et al., (2008) concluded in his findings on extraction of penicillin G that while the results point to ionic interactions as being the main controlling factor in the extraction of protein in reversed micelles and at higher pH, the decrease in the amount of penicillin G extracted might be due to denaturation of penicillin G. Hence, this study suggests that the optimum pH to be used in further steps is 6.0, since the highest amount of extracted jacalin was obtained at that pH value.

4.6.3 Overall Mass Transfer Coefficient of the Extraction in Reverse Micelle

Reversed micelles extraction is a kinetic process in which solvents are transferred to and from reversed micelles. During the forward extraction process, jacalin molecules are diffused from the the aqueous phase to the interface between aqueous and organic phase, then encapsulate the jacalin. The reversed micelle aggregates containing jacalin are further diffused from the interface into the bulk organic phase, and vice versa for the backward extraction. The process reaches a kinetic equilibrium at the end of the extraction (Figure 4.19).

The mass transfer of jacalin in both forward and back extraction can be described by the two-film theory combined with interfacial resistance (Liu et al., 2006; Lye et al., 1994; Setapar et al., 2012). The majority of studies on protein extraction using reversed micelles system have focused on the thermodynamic behavior of the microemulsion phase. Equilibrium studies are crucial to the optimization of reverse micelle extraction and to the understanding of the parameters that affect protein solubilisation in these systems. It is important to have information about the rates of forward and backward transfer of the protein into and out of the reverse micelle across the oil-water interface. Knowledge of these rates is required to determine whether interfacial transport is rate limiting relative to bulk transport processes. The nature of the transfer of proteins in reversed micelles is at present unknown, with possible contributions from different forces (chemical, electrostatic, hydrophobic, and flux dynamics interactions) acting between the solute and the interface (Melo et al., 2001).

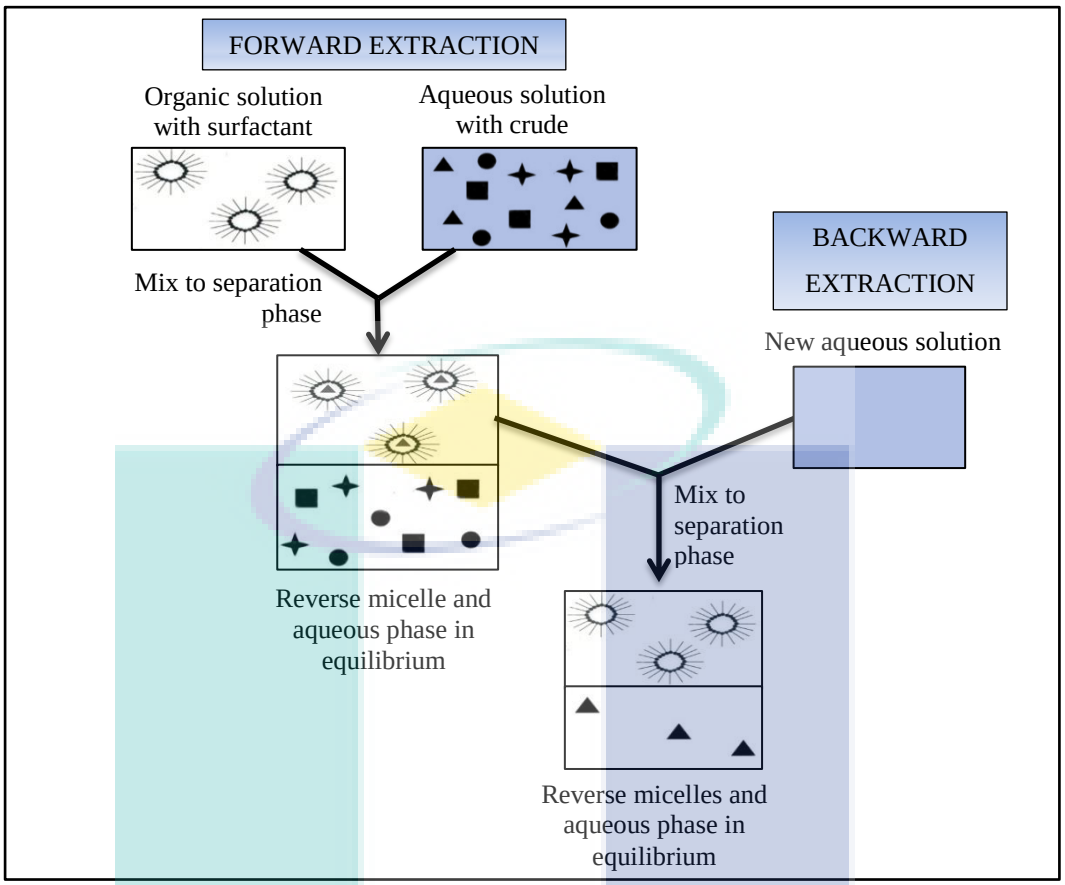


Figure 4.19: Reversed micelles of protein extraction are based on a forward and a back extraction to a new aqueous phase.

The mass transfer rates of jacalin and water solubilisation rate were determined for the batch extraction. These rates were measured for various protein concentrations and times. There are diffusional resistances for transport from an aqueous phase to a reversed micelle organic phase and in the organic phase into aqueous phase. Therefore, by shaking both upper and lower phases, this resistance was reduced to that operating in the boundary layer region adjacent to the interface. The effect of shaking in an incubator shaker was therefore to decrease bulk transport limitations by reducing the thickness of the boundary layer (Dövyap et at., 2006). Kinetic parameters for the transfer of protein to or from a reversed micelle solution were determined at a given salt concentration and pH by measuring the protein bulk concentration as a function of time. Samples were taken from the reversed micellar phase for forward transfer experiments and from the aqueous phase for back transfer experiments. In all cases, the rates were determined from short-time data in which the flux was a constant. This initial flux J is related to the

mass transfer coefficient, as expressed through the following equations (Dungan et al., 1991 and Hebbar et al., 2011):

$$J = \frac{V}{A}\frac{dc_{meas}}{dt} \tag{4.7}$$

$$J = k_f\left(C_{aq} - \frac{1}{H}C_{org}\right) \tag{4.8}$$

$$J = k_b\left(C_{org} - HC_{aq}\right) \tag{4.9}$$

Eq. (4.7) and (4.8) are for forward and backward extraction, respectively, where k_f and k_b are the mass transfer coefficients for forward and back transfer respectively, C_{aq} and C_{org} are concentration of aqueous and organic phase, V is the volume of each phase and A is the interfacial area. In this equation H is the partition coefficient and $H = C_{org}/C_{aq}$ relates to the equilibrium concentrations in each phase. Given that for most forward transfer conditions studied, $C_{org}/H \ll C_{aq}$. The overall mass transfer coefficient, ka min^{-1} for forward and back extraction, was estimated using the equations proposed by Hebbar et al. (2011). The relationships for the transfer of protein from the aqueous phase to the organic phase can be written as:

$$\ln\left(1 - C_{org}/C_{aq}^{0}\right) = -\left(A/V\right)k_{f0}t \tag{4.10}$$

$$\ln\left\{\left(1 - \left(C_{org}/C_{aq}^{0}\right)\right)\right\} = -\left(A/V\right)k_{f0}t = k_{f0}at = K_f t \tag{4.11}$$

$$\ln\left\{\left(1 - \left(C_{org}/C_{aq}^{0}\right)\right)\right\} = -\left(A/V\right)k_{b0}t = k_{b0}at = K_b t \tag{4.12}$$

Where C_{org} and C_{aq} are the concentrations of the solute in the organic and aqueous phases, superscript ‘0’ indicates the initial concentration, ‘A’ is the interfacial area, ‘V’ is the volume of phase, and the ‘t’ is the phase mixing time. Subscripts ‘f’ and ‘b’ indicate forward and back extraction, respectively. ‘K’ is the overall mass transfer coefficient. Thus, the mass transfer coefficient could be evaluated from the slope of the left-hand side, plotted as a function of time.

To study the mass transfer kinetics during forward extraction, phase mixing was carried out at different time intervals, and protein concentrations were estimated for studies at 15 mL phase volume. To estimate the forward extraction mass transfer coefficient, a plot of $\ln\{1-(C_{org}/C_{aq}^{0})\}$ against mixing time was drawn for the initial period of mixing (30 min), during which maximum extraction took place (Figure 4.20). The mass transfer coefficient K_f was estimated from the slope of the line drawn. Since it is difficult to estimate the interfacial area or contact area under mixing conditions, the mass transfer coefficient was estimated in terms of $k_{fo}a$ (min^{-1}). The estimated mass transfer coefficient of forward extraction was found to be 0.0256 min^{-1}.

Back transfer experiments were carried out using the reversed micelles phase obtained from forward extraction. For all the experiments, forward extraction time was maintained at 30 min. Back extraction was carried out at different time intervals and the corresponding protein concentrations in the aqueous phase were measured. Like in the case of forward extraction, nearly 43 % of the extraction took place in the first 30 min. Practically, there was no extraction of solute observed after 30 min. The mass transfer coefficient for back extraction (Figure 4.21) was estimated from the plot of $\ln\{1-(C_{org}/C_{aq}^{0})\}$ against time and it was found to be 0.0183 min^{-1}, which is lower than that obtained for forward extraction. Dungan et al., (1991), Dong et al., (2009) and Hebbar et al., (2011) reported the back extraction rate to be three to four times slower than the forward transfer, which indicates that interfacial resistance plays a more important role in back extraction than in forward extraction. This is possibly due to greater interfacial resistance, hence more difficulty in releasing proteins at the oil–water interface in back extraction (Liu et al., 2006).

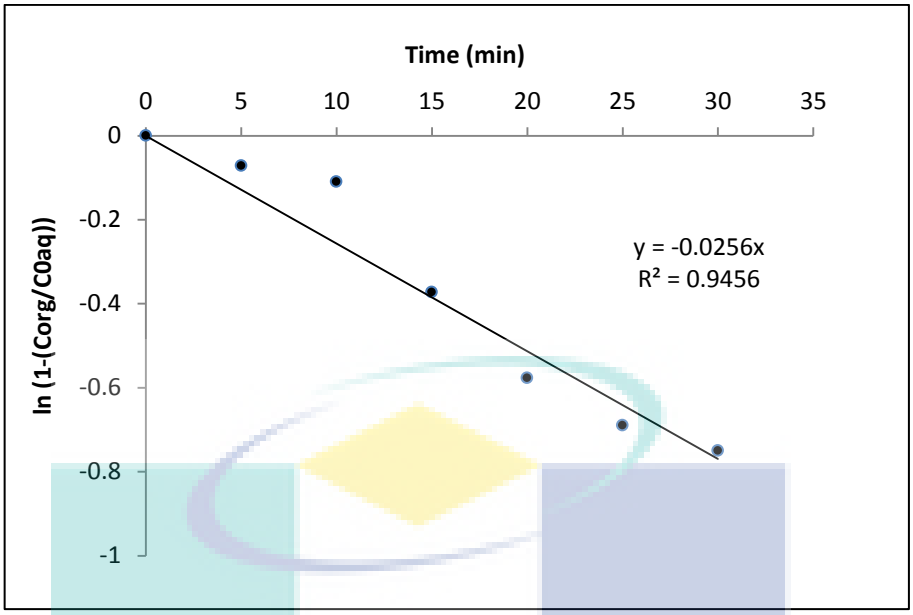


Figure 4.20: Estimation of mass transfer coefficient for forward extraction of jacalin. A plot of $\ln\{1-(C_{org}/C_{aq}^{0})\}$ against mixing time was drawn for the initial period of mixing (30 min), and $k_{fo}a$ (min^{-1}) was 0.0256.

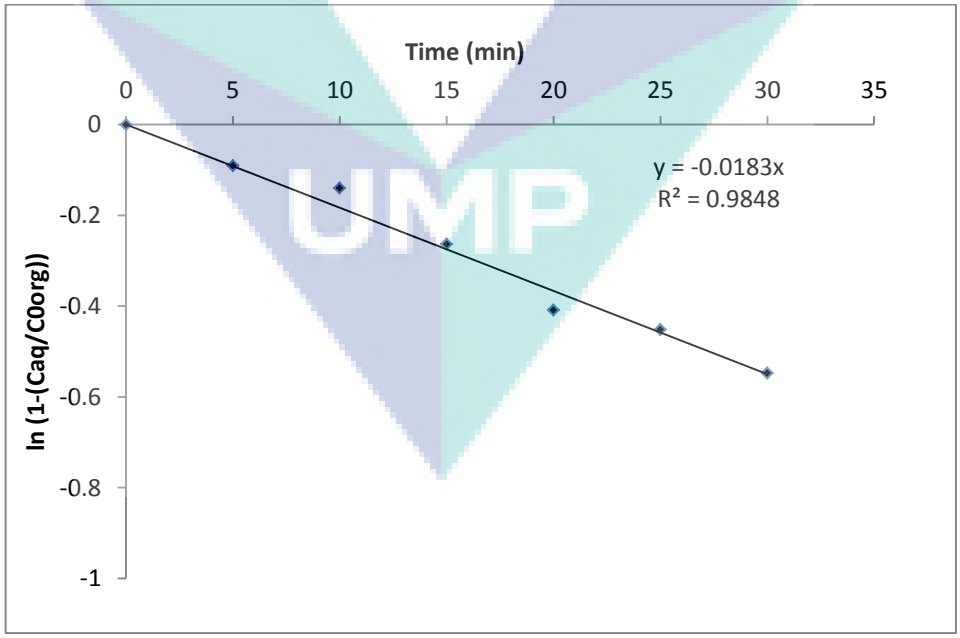


Figure 4.21: Estimation of mass transfer coefficient for back extraction of jacalin. From the plot of $\ln\{1-(C_{org}/C_{aq}^{0})\}$ against time and it was found to be 0.0183 min^{-1}, which is lower than that obtained for forward extraction.

4.6.4 Conclusion of Extraction using Reverse Micelle

The reversed micelles system was successfully used to extract jacalin from the crude protein of jackfruit seed powder. The experiment was conducted using AOT as surfactant, NaCl as salt to control ionic strength and pH level was studied in forward extraction. The results show that the optimum pH to be used in further jacalin extraction is 6.0, since the highest amount of jacalin extracted was obtained at that pH value. Otherwise, salt concentration up to 0.2 M was just sufficient to enhance the interaction between jacalin and surfactant head to reduce the repulsive forces between the two surfactant heads, resulting in the optimum level of extracted jacalin in the forward extraction. In conclusion, the most optimal values of variables possible that can enhance extraction of jacalin are pH at 6.0 and salt (NaCl) concentration at 0.2 M, contributing to the highest yield of 1.78 mg/mL. It was noticed that pH and salt concentration influenced the extraction of jacalin from the crude protein of jackfruit seed powder.

Othman et. al., (2006) used affinity chromatography with ammonium sulfate to produce 11 to 22 % of jacalin extracted from the crude protein of jackfruit seed powder. Meanwhile, Roy et. al., (2005) used cross-linked guar gum method in the extraction of jacalin and produced 1.67 % of jacalin. Other than that, Kabir (1995) collected 20 mg of jacalin from 200 mg of crude protein, which means 30 % of jacalin was extracted from crude protein using chromatography with galactose column. Compared to this study, only 5 % of jacalin was extracted from crude protein of jackfruit seed powder using reversed micelles extraction method. However, reversed micelles extraction method is better than cross-linked guar gum in terms of jacalin yield. Furthermore, chromatography with expensive column type is rather costly. The use of reversed micelles reported here illustrates a one-step interaction strategy with a much cheaper chromatographic column (Roy, 2005).

The study of the mass transfer coefficient of this method may improve the production of extracted jacalin. The experimental kinetic data was fitted to a theoretical equation; the mass transfer rates for both the forward and the back extraction in reversed micelles extraction method were obtained and compared with literature reports of protein extraction. The results show that back extraction is slower than forward

extraction, which indicates that the interfacial resistance plays a more important role in back extraction than in forward extraction. The results were equivalent to previous kinetic studies of protein extraction.

4.7 CELL LINES BIOACTIVITY

This section presents the result of the bioactivity study of jacalin extracted from crude protein of jackfruit seed powder (JSP) on the growth of two human cancer cell lines, non-small lung carcinoma (H1299) and breast cancer (MCF7), as evaluated by MTT assay. Jacalin is a lectin from *Artocarpus*, which is a valuable source of medicinally important compounds, besides being an edible fruit rich in minerals, vitamins, antioxidants and other nutrients (Jagtap and Bapat, 2010). This study was focused on its cytotoxicity due to the abundant amount of the sample (jackfruit seed). The MTT assay is a common practice for studying the action of natural products on cell viability and cytotoxicity. This assay is based on reduction of tetrazolium salt to a purple insoluble formazan by metabolically active cells (Bakar et al., 2010). The absorbance of the solubilized formazan is taken as a measure of the number of living cells.

4.7.1 The Cytotoxic Effects on Non-small Lung Carcinoma Cells

The evaluation of cell viability against non-small lung carcinoma (H1299) cells was conducted at different concentrations. MTT cell viability assay was used to analyse the effects of different types of samples (crude protein, jacalin extract and standard jacalin) on H1299 cancer cell lines. Figure 4.22 presents a comparison between cell viability affected by crude protein and jacalin extracted from jackfruit seed powder against H1299 viability cell and compared with jacalin standard. The evaluation of cell viability was studied after 72 hr exposure with concentrations of samples at 50 and 100 µg/mL.

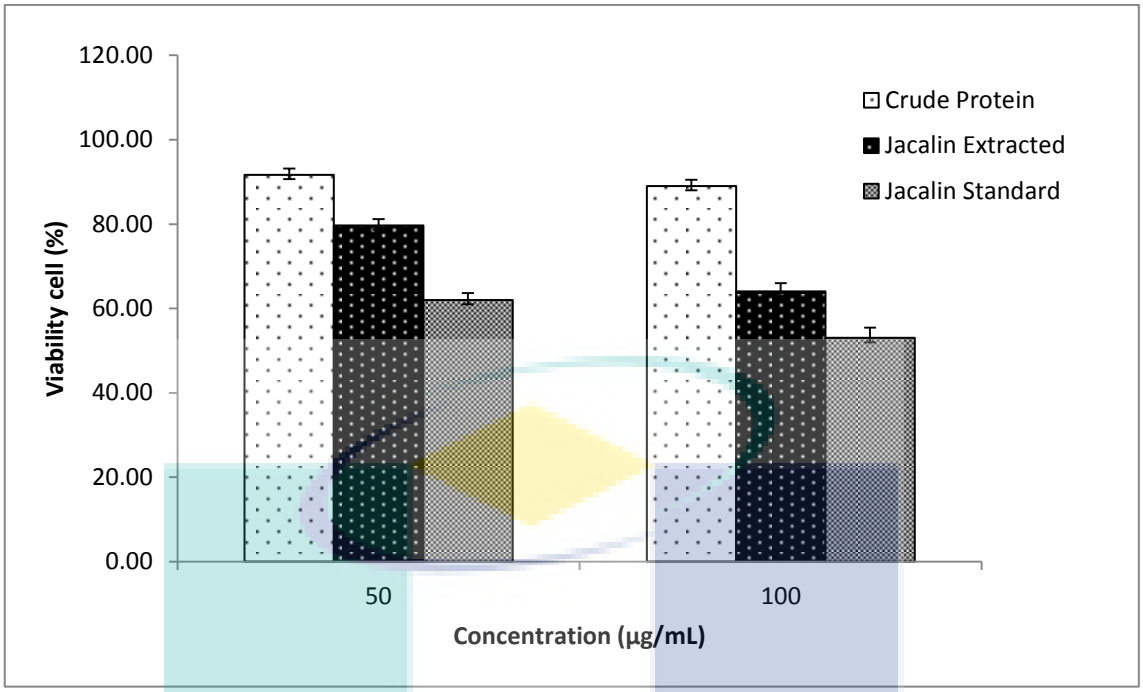


Figure 4.22: The effect of crude protein, jacalin extract and jacalin standard on the viability of H1299, non-small lung carcinoma cell, determined using MTT assay at 50 and 100 µg/mL concentration. The results were examined based on cells with a density of $1x10^5$ cells/mL, treated for 72 hr in a 96-well plate.

The graph shows a dropping trend line of cell viability for crude protein and jacalin extract parallel with jacalin standard. However, the comparison between the reducing rate of crude protein and jacalin extract was much different. The reducing rate of crude protein was 2.91 %, whereas for jacalin extract it was 7.95 %, from 50 to 100 µg/mL of concentration range. The improvement in inhibition of H1299 cell was 5.04 % higher in jacalin extract compared to crude protein. This shows that extraction of jacalin by reversed micelles liquid-liquid extraction had succeeded in selecting lectin to inhibit the H1299. Other than that, this result also supports that the jacalin extract was a similar lectin to the jacalin standard in regards to having the same trend of effect towards H1299 cell.

A previous research on lung cancer by Lu et al., (2013) studied on how adlay seeds interfere with the growth, metabolism and proliferation of non-small cell lung cancer, thus finding that adlay seeds lead to the induction of apoptosis in these cancer cells. The mechanism of apoptosis induction in cancer cells is worthy of further investigation because of its potential anti-cancer effect. Besides that, the findings by Cui et al., (2014) suggest that polysaccharides derived from edible fungi can inhibit non-small cell lung cancer proliferation and induce apoptosis mainly by activating the intrinsic mitochondrial pathway.

4.7.2 The Cytotoxic Effects on Human Breast Cancer Cells

The cytotoxic effects of lectin from jackfruit seed (crude protein and jacalin extract) on the viability of human breast cancer cell (MCF7) was evaluated by MTT assay. Figure 4.23 shows the cytotoxic effects examined at 50, 100 and 200 µg/mL concentration of sample and compared with jacalin standard. Meanwhile, in Figure 4.24, a graph of percentage of cell viability was plotted to determine the concentration of 50 % inhibition of cell viability, also known as IC_{50}. The inhibitory concentration required to reduce 50 % of cell viability (IC_{50}) was then compared to the untreated control. The results were calculated based on treated MCF7 cells with a density of $1x10^5$ cells/mL, treated for 72 hr in a 96-well plate with crude protein as well as jacalin extract, and compared with jacalin standard.

Figure 4.23 shows that the crude protein did not have much effect on the viability of MCF7 cell, as 50, 100 and 200 µg/mL concentrations of sample gave the result of 92, 89 and 86 % cell viability, respectively. Overall, there was only a minor decrease in cell viability (dropping only 17 %) with crude protein, and it may be assumed that it would not achieve IC_{50} for inhibition of this cancer cell inhibition. Whereas jacalin extract showed a large drop from 200 to 50 µg/mL, which is a 92 % drop in cancer cell viability. The results of the jacalin extract show an almost similar trend to jacalin standard, whereby jacalin standard also had a large decrease in cell viability (93 %). From this result it can be concluded that the jacalin extract was better than crude protein in inhibiting the viability of MCF7 cancer cell.

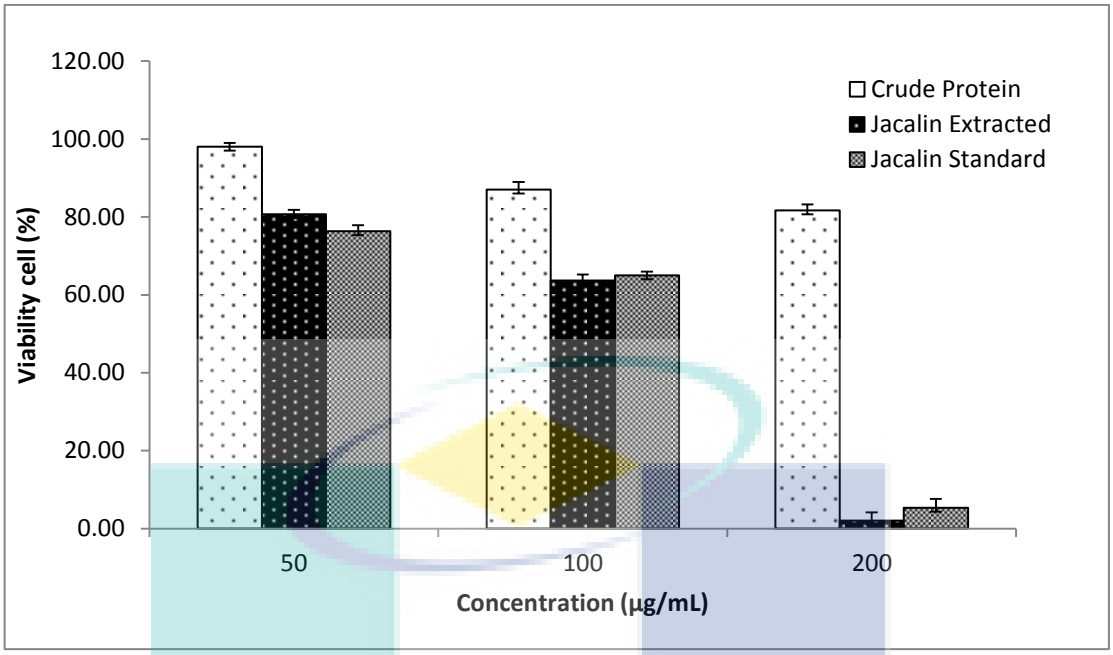


Figure 4.23: The effects of crude protein, jacalin extract and jacalin standard on viability of MCF7, human breast cancer cells were examined at 50, 100 and 200 µg/mL concentration.

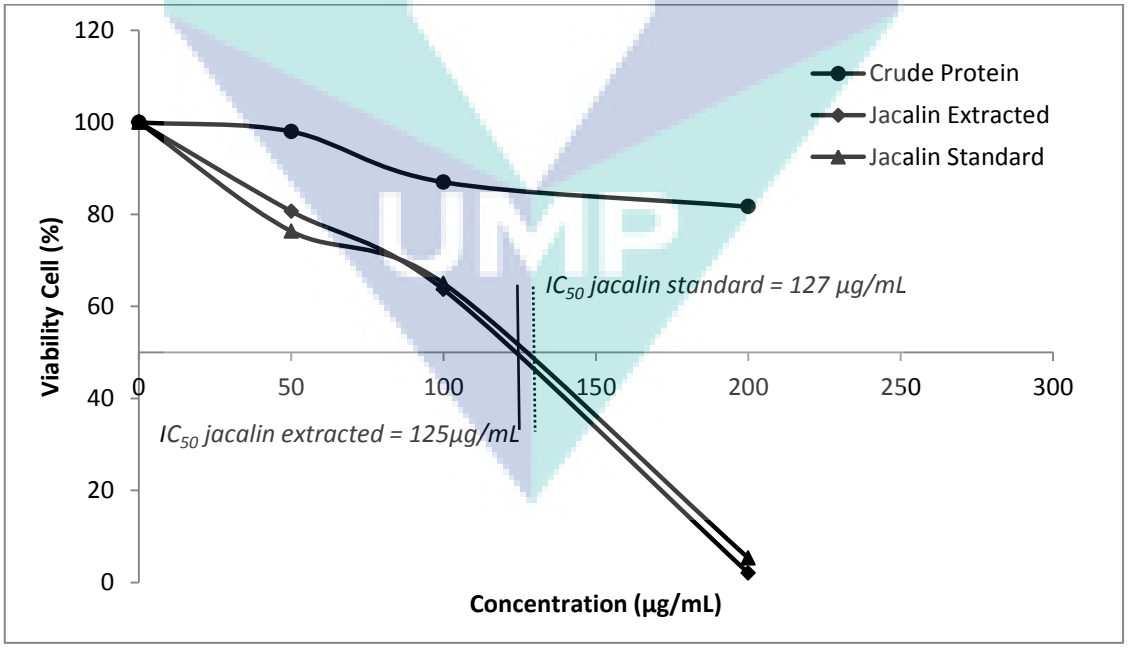


Figure 4.24: A graph plotting the percentage of cell viability versus concentration of jacalin extract and the concentration that gave 50 % inhibition of cell viability (IC_{50}) was determined for breast human cancer cell (MCF7).

As shown in Figure 4.24, cytotoxic effects towards MCF7 cell reached IC_{50} for jacalin extract and jacalin standard at concentrations 125 and 127 µg/mL, respectively. This is dissimilar to crude protein, which still did not reach IC_{50} even when extended till 200 µg/mL concentration. This means extraction of crude protein using reversed micelles was selective in filtering compounds, contributing to the cytotoxic effect towards MCF7 cell. On the other hand, the jacalin extract result was parallel with jacalin standard, so it can be concluded that the jacalin extract has the same biological function as jacalin standard.

The overall results in Figure 4.23 and 4.24 shows that jacalin extract had better inhibition effect on MCF7 cancer cells compared to crude protein. Jacalin was extracted by reversed micelles liquid-liquid extraction, which was selective for lectin which had the effect of inhibiting MCF7 cells. Besides that, from this result it can be concluded that the extract contained jacalin since its biological effect on MCF 7 cancer cell was similar to jacalin standard. This is related to a previous study by Kabir (1998), who mentioned that jacalin has been used to study tissue binding properties in breast and thyroid cancer cell.

4.7.3 Conclusion of Application of Jacalin to Cancer Cell Lines

The results of the present study have demonstrated that crude protein and jacalin extracted from JSP inhibit the cell viability for two types of cancer cells, MCF7 and H1299. However, crude protein had a slowed down trend of decreasing cell viability for both types and did not reach IC_{50}. Therefore, it was not worth it to raise its concentration and instead it was recommended to extract jacalin from the crude protein prior to treatment. Through detailed *in vitro* study studies, the results propose that lectin derivatives from JSP called jacalin have the potential to inhibit the growth of cancer cells. These findings are parallel to the suggestion by Kabir (1998) that jacalin has potential as a therapeutic agent for cancer and it has been used as a histochemical reagent to study tissue binding properties in benign and malignant lesions of the breast and the thyroid.

Upon determining the inhibitory effect of jacalin on cancer cells (MCF7 and H1299), further study identified that jacalin extract was more effective compared to crude protein in treatment towards reducing cell viability. The reason for this is that crude mixtures of lectin are complicated and unknown in their details compared to extracts with selective and filtered compounds. Other than that, lectins are involved in cell recognition and aggregation (Pajic et al., 2002), but all biological functions of lectins in these organisms have not yet been determined. The mechanism of the cytotoxic activity of jacalin is still unknown, and it could reveal some new and interesting facts about the role of lectins as a treatment agent in prohibiting the viability of cancer cells. Meanwhile, a report by Sahasrabuddhe et al., (2006) found that native jacalin from *A. Intergrifolia* seed was a cytotoxic inhibitor of proliferation of epidermoid carcinoma (A431) and human colorectal carcinoma (HT29), and suggested that jacalin induces cytotoxicity by facilitating protein-protein interactions that impair the functions of the cell, thus making the cell more susceptible to death due to stress. It has been proved that jacalin can be a prominent agent in cancer cell treatment today as well as in the future. Furthermore, the haemagglutinating activity of jackfruit seed protein was found to be specifically and most effectively inhibited by N-acetyl galactosamine, and to a lesser degree by galactose, therefore suggesting that the agglutinating factor is probably a lectin specific for *N*-acetyl galactosamine (Laija et al., 2010).

4.8 ANALYSIS OF PRODUCT

4.8.1 Estimation of Group Compound

FTIR spectroscopy analysis was conducted to study the chemical structure and identify the biomolecules that were bound specifically on the protein compound of crude protein, jacalin extract and jacalin standard. Figure 4.25 and Table 4.16 show a shift in 4 peaks for crude protein at 3341, 2123, 1630 and 1077 cm^{-1}. Meanwhile, jacalin extract has 3 peak waves at 3351, 2119 and 1636 cm^{-1}. Thus, the FTIR analyses indicate that these are possibly bonds due to N-H stretching (amine), C═C stretching (alkynes), N-H bending (amide) and C-N stretching (aliphatic amine). The wave peak at 1630 cm^{-1}

shows the presence of the carbonyl group (C-O) in stretching mode associated with amide bond (Kumar et al., 2013). These FTIR analysis findings for functional groups such as NH and CO are similar to findings on functional groups for jackfruit leaf powder by Uddin et al., (2009).

Furthermore, jacalin extract has 3 peak waves at 3351, 2119 and 1636 cm^{-1}, which is practically similar to jacalin standard peaks at 3343, 2113 and 1636 cm^{-1}, which means that both of them have the same group compounds. Therefore, this finding proves that the jacalin extracted from crude protein using reversed micelles liquid-liquid extraction is a jacalin compound that has exactly similar group compounds with jacalin standard. Comparing the group compounds by peaks raised in Figure 4.25 for crude protein and jacalin extract, these graphs show that jacalin extract is missing one peak (1077 cm^{-1}), representing the aliphatic amine group with C-N stretching. According to Manara et al., (2014), the peak around 1600 and 1598 cm^{-1} in all the lignin samples are characteristic of aromatic ring vibrations of the phenylpropane groups and it is caused by the impact of extraction conditions on chemical properties during lignin isolation. Hence, that group compound possibly separated during the reversed micelles liquid-liquid extraction process. It can be concluded that the reversed micelles extraction method can be used as a purification method for jacalin extracted from crude protein of jackfruit seed powder. Many previous studies have used reversed micelles as a purification method, such as purification of lipase by Nandini and Rastogi, (2009), purification of tannase by Gaikaiwari et al., (2012), and purification of lectin from black turtle bean (*Phaseolus vulgaris*) by (He et al., 2015).

Table 4.16: Comparing a shift in peaks of compound group for crude protein, jacalin extract and jacalin standard.

Peak No.	Crude Protein		Jacalin Extract		Jacalin Standard	
	Protein Frequency	Intensity Value	Protein Frequency	Intensity Value	Protein Frequency	Intensity Value
1	3341.71	0.23	3351.26	0.26	3334.3	0.25
2	2123.86	0.02	2119.72	0.04	2113.00	0.04
3	1636.20	0.11	1636.01	0.13	1636.37	0.12
4	1077.34	0.05				

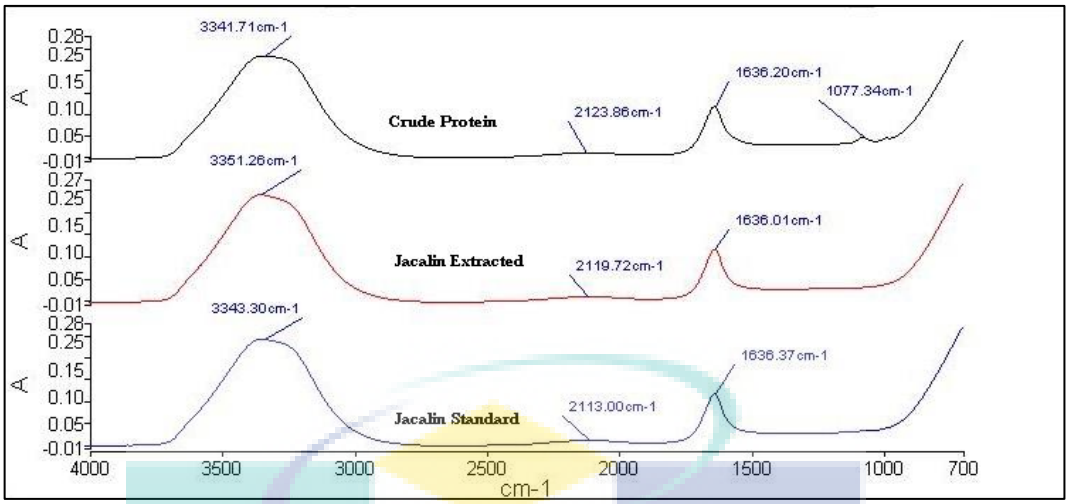


Figure 4.25: Comparing a shift in peaks of compound group for crude protein, jacalin extract and jacalin standard. This was recorded using Varian 660 FTIR spectrometer in the 4000-700 cm^{-1} region (resolution of 4 cm^{-1}) and 16 scans per spectrum in transmittance mode.

4.8.2 Estimation of Molecular Weight

The crude protein and the jacalin extract were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 10 % gel. The purity of the jacalin extract was ascertained by SDS-PAGE profiles (Figure 4.26), which indicate molecular weight markers (lane 1) with crude protein (lane 2), jacalin extract (lane 3) and jacalin standard (lane 4). The crude protein shows a large number of bands, while jacalin extracted by reversed micelles extraction clearly shows two bands at 15 and 17 kDa, similar to jacalin standard. The reduction in the number of bands indicate that the extraction of jacalin from crude protein and the band obtained lie close to jacalin standard. This indicates that the extracted jacalin could be successfully separated and purified using reverse micelles extraction. The reduction in the number of bands in SDS-PAGE was due to negligible recovery of other proteins. Other than that, the SDS PAGE results for jacalin extract show two bands at approximately 15 to 17 kDa, which is consistent with previous studies by Young et.al, (1989), Kabir, (1995) and Pratap et.al, (2002) that reported jacalin was observed at molecular weights within 11 to 17 kDa.

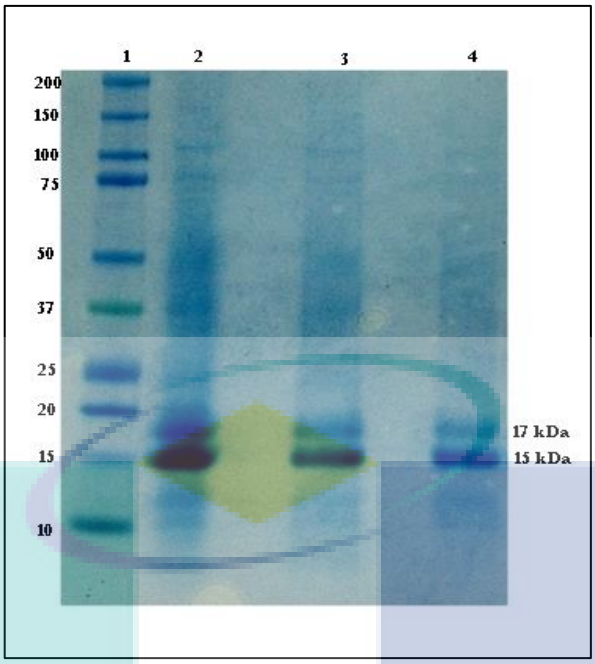


Figure 4.26: SDS–PAGE analysis of reversed micellar extracted jacalin extracted from crude protein of jackfruit seed powder. Lane 1: molecular marker; Lane 2: crude protein; Lane 3: jacalin extract and Lane 4: jacalin standard

4.8.3 Protein Identification

The identification of lectin from JSP (crude protein and jacalin extracted) used mass spectrometry analysis of MALDI-TOF/TOF by ABICIEX, USA. Then, the analysis profile of the peptide fragment was analyzed using the MASCOT database search engine ABSCIEX protein pilot software, and spectrum results are shown in Figure 4.27. Meanwhile, Table 4.17 shows a summary of protein types in crude protein, jacalin extract and jacalin standard. The results indicate two types of proteins with high score (more than 60), which are leca artin and leca artto, with mass 14653 Da and 2851 Da, respectively. Protein sequence was coding as AFDDGAFTGIR.E with means Ala-Phe-Asp-Asp-Gly-Ala-Phe-Thr-Gly-Ile-Arg.Glu. As can be seen from Figure 4.27, the separation spectrum of protein of jacalin extract is practically similar to jacalin standard, in contrast to crude protein.

Table 4.17: List of peptide in crude protein, jacalin extract and jacalin standard

No.	Type of Protein	Mass (Da)	Score	Protein
Crude Protein				
1.	LECA ARTIN Agglutinin alpha chain (Artocarpus integer)	14653	115	K.AFDDGAFTGIR.E
2.	LECA ARTTO Agglutinin alpha chain (Artocarpus tonkinensis)	2851	138	K.AFDDGAFTGIR.E
Jacalin Extracted				
1.	LECA ARTIN Agglutinin alpha chain (Artocarpus integer)	14653	63	K.AFDDGAFTGIR.E
2.	LECA ARTTO Agglutinin alpha chain (Artocarpus tonkinensis)	2851	63	K.AFDDGAFTGIR.E
Jacalin Standard				
1.	LECA ARTIN Agglutinin alpha chain (Artocarpus integer)	14653	93	K.AFDDGAFTGIR.E
2.	LECA ARTTO Agglutinin alpha chain (Artocarpus tonkinensis)	2851	88	K.AFDDGAFTGIR.E

4.8.4 Conclusion of Properties of Jacalin

Overall, the results of the analysis of molecular weight by SDS-PAGE analysis, group compound by FTIR and MASCOT protein identification by MALDI-TOF/TOF determined that the jacalin extract was similar to jacalin standard. Furthermore, results of cytotoxicity towards MCF7 and H1299 cancer cells show a similar biological function for jacalin extract and jacalin standard. It is possible to conclude that the extraction of jacalin from crude protein using reversed micelles liquid-liquid extraction method was successful in separating and filtering jacalin from the crude protein. Therefore, the reversed micelles extraction method is suggested for used in further studies as an alternative method for purification of jacalin from crude protein.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In general, the study was carried out to examine the recovery of crude protein from jackfruit seed powder (JSP). In determination of the optimum recovery process, the effects of liquid to solid ratio, temperature and recovery time on the concentration of recovered crude protein were examined. However, prior to studying the effects of the parameters, the best soaking pretreatment time was determined. Upon determination of the best soaking pretreatment time and optimum recovery condition, study was further conducted to examine the highest extraction yield of jacalin from crude protein. The extraction of jacalin in this study used reversed micelles liquid-liquid extraction. The main conclusions of this study are summarized as follows:

1) Soaking pretreatment was identified as one of the important factors in crude protein recovery of jackfruit seed powder. Based on the optimum crude protein concentration, 6 hr of soaking was selected as the finest pretreatment time.

2) In studying the effects of independent variables by OFAT approach on crude protein recovery from jackfruit seed powder, three independent variables, namely LSR, temperature and recovery time were evaluated.
   i) Crude protein concentration recovered from JSP was significantly decreasing when LSR are increasing and the highest crude protein concentration being 29.03 mg/mL at 3 mL/g.
   ii) Crude protein concentration increasing parallel with temperature and reached the highest concentration of 25.81 mg/mL at 31 ^{o}C.

iii) Crude protein concentration sharply increased with recovery time from 0 to 6 hr with concentrations 17.06 mg/mL and 33.18 mg/mL, respectively.

3) Optimization by response surface methodology (RSM) was obtained:
   i) the crude protein concentration was 29.11 % greater than the values before optimization, hence the optimum crude protein concentration could be achieved using RSM.
   ii) the value of crude protein concentration from the model was 44.20 mg/mL and the experimental results was 42.84 mg/mL, which percentage errors from 7.42 to 8.20 (within the value of 10 %), indicating that the empirical models developed were successfully validated and accepted the model.

4) Reversed micelles system was successfully used to extract jacalin from crude protein of jackfruit seed powder with optimum concentration 1.78 mg/mL at pH 6.0 and salt concentration up to 0.2 M.

5) The estimated mass transfer coefficient of forward extraction was found to be 0.0256 min^{-1}, and 0.0183 min^{-1} for back extraction. The results show that the back extraction is 28.52 % slower than the forward extraction.

6) In the cytotoxicity study of the inhibitory effect of jacalin on cancer cells (MCF7 and H1299), it was identified that jacalin extract was more effective compared to crude protein in treatment against cell viability. It was 28.01 % and 26.81 % more effective compared to crude protein toward MCF7 and H1299 cancer cells, respectively.

7) The cytotoxicity study found the jacalin extract was more effective towards MCF7 cancer cell compared to H1299, with 63.67 and 73.30 % cell viability respectively. IC_{50} was achieving at 125 µg/mL concentration of jacalin extract, nearly similar to jacalin standard IC_{50} at 127 µg/mL.

5.2 Recommendations

Recommendations are suggested for future work which can be performed to give better understanding and improvement on the extraction of jacalin from jackfruit seed powder. Below are some recommendations for future work:-

1) Expected crude protein recovery can be maximised in a continuous process using the optimum conditions found in this research. The continuous process is to ensure crude protein is completely recovered.

2) The expected residue from recovery of jackfruit seed powder can be used as a by-product in the animal food industry or others.

3) The separation process using reversed micelles is based on an ordinary liquid-liquid extraction technique, it is easy to scale up and operate continuously. Therefore, it is worth scaling up the extraction of jacalin from crude protein to achieve the current market demands for jacalin.

4) Extend the study of parameter will effect of the crude protein recovery process such as particle size of jackfruit seed powder and/or different species of jackfruit seed.

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APPENDIX A

LIST OF CHEMICALS

No.	List of Chemical	Supplier
1	Dulbecco's Modified Eagle Medium (DMEM)	GIBCO
2	Fetal bovine serum (FBS)	GIBCO
3	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit	Introgen (USA)
4	Phosphate and carbonate buffers	Sigma Chemical Co.
5	Phosphate buffered saline (PBS) tablet	Sigma
6	Dimethylsufoxide (DMSO)	Sigma
7	Bovine Serum Albumin (BSA)	Sigma
8	Sodium di-2-ethylhexylsulfoccinate (AOT)	MERCK
9	Kalium Chloride (KCl)	MERCK
10	Sodium Chloride (NaCl)	MERCK
11	Sodium hydroxide (NaOH)	MERCK
12	Sodium potassium tartarate ($KNaC_4H_4O_6 \cdot 4H_2O$)	MERCK
13	Folin-Ciocalteau reagent	MERCK
14	Isooctane ($(CH_3)_3CCH_2CH(CH_3)_2$)	MERCK
15	Hydrochloride acid (HCl)	MERCK
16	Potassium dihydrogen phosphate (KH_2PO_4)	MERCK
17	Potassium hydrogen phosphate (K_2HPO_4)	MERCK

APPENDIX B

PREPARATION OF BUFFER SOLUTION

Potassium phosphate buffer, 0.01M

Solution A: 2.72 g KH_2PO_4 per liter (0.02 M)

Solution B: 4.56 g K_2HPO_4 per liter (0.02 M)

Referring to Table A2-1 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 B-1: Preparation of 0.01M 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

APPENDIX C

PREPARATION OF LOWRY ASSAY

1) Reagent to be prepared

Solution A : Stock solution 1 mg/mL of Bovine Serum Albumin (BSA)

Solution B : 50 mL of 2% sodium carbonate mixed with 50 mL of 0.1 N NaOH solution (0.4 g in 100 mL distilled water)

Solution C : 10 mL of 1.56 % copper sulphate solution mixed with 10 mL of 2.37 % sodium potassium tartarate solution.

Solution D : Is Lowry reagent by mixing 2 mL of (Solution B) with 100 mL of (Solution A)

Solution E : Folin-Ciocalteau reagent solution dilute with an 6 volume of water on the day of use

2) Procedure for analysis by Lowry method

i) Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/mL) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 mL. The BSA range is 0.05 to 1 mg/mL.

ii) From these different dilutions, pipette out 0.2 mL protein solution to different test tubes and add 2 mL of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well.

iii) This solution is incubated at room temperature for 10 min.

iv) Then add 0.2 mL of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660 nm.

v) Plot graph the absorbance against concentration to have a standard calibration curve.

vi) Check the absorbance of unknown sample and determine the concentration of the unknown sample using the standard curve plotted above.

Table C-1: Bovine serum albumin concentration range to set up standard curve

BSA (mL)	Water (mL)	Alk.$CuSO_4$ (mL)	Lowry reagent
0.00	1.00	2	0.2
0.20	0.80	2	0.2
0.40	0.60	2	0.2
0.60	0.40	2	0.2
0.80	0.20	2	0.2
1.00	0.00	2	0.2

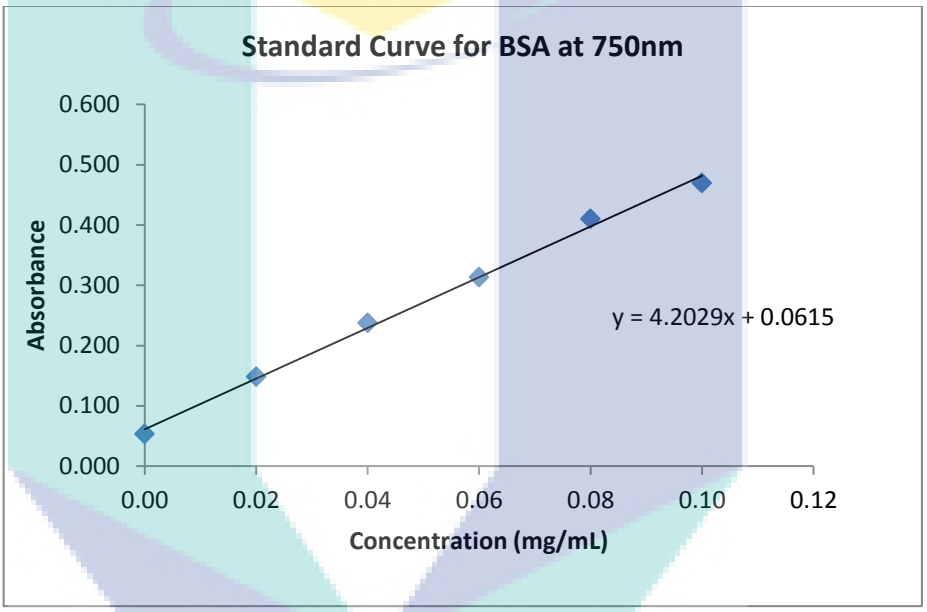


Figure C-1: Example of standard curve graph for BSA at 750 nm wavelength

APPENDIX D

PREPARATION OF CYTOTOXICITY ASSAY

1) Seeding the cell lines in 96-well plate (A) at a density of $1x10^5$ cells/mL
2) Seeding cells in well plate (A) were incubated at 37°C for 24 hr or until cells confluence completely.
3) Preparing of series dilution of sample in DMEM media in 96-well plate (B)
   - 20 µL sample mixed 180 µL DMEM media to produced concentration of 100 µg/mL sample in media.
   - 100 µL of first dilutions (100 µg/mL) was mix with 100 µL of fresh DMEM to be concentration sample in media was 50µg/mL.
   - 100 µL of second dilutions (50 µg/mL) was mix with another 100µL of fresh DMEM to produced concentration of 25 µg/mL sample in media.
   - 100 µL of third dilutions (25 µg/mL) was mix with another 100µL of fresh DMEM to formed 12.5 µg/mL of sample in media.
   - 100 µL of forth dilutions (12.5 µg/mL) was mix with 100 µL of fresh DMEM to made 6.25 µg/mL of sample in media.
   - 100 µL of firth dilutions (6.25 µg/mL) was mix with another 100 µL of fresh DMEM to be 3.125 µg/mL.
4) Confluence cell in 96-well plate (A) was taken out the medium and washed with PBS twice to replace with fresh medium (whereas prepared with sample as in point 3) to provide complete nutrient for cell along the incubation with sample during treatment process.
5) A series dilution of sample in 96-well plate (B) was pipet into seeding cell in 96-well plate (A) in triplicate and was incubated at 37 °C for 72 hr.
6) The medium was then discarded and the adherent cells were washed twice with phosphate buffer solution (PBS)
7) 20 µL of MTT stock solution (5 mg/mL in PBS) were added to each well and the plates were further incubated for 4 hr at 37 °C.
8) 100 µL of dimethylsufoxide (DMSO) was added to each well to solubilize the formazan crystals produced by viable cells.

9) After formazan blue was completely dissolved, absorbance was measured at 570 nm wavelength, using TECAN infinite M200 micro plate reader.

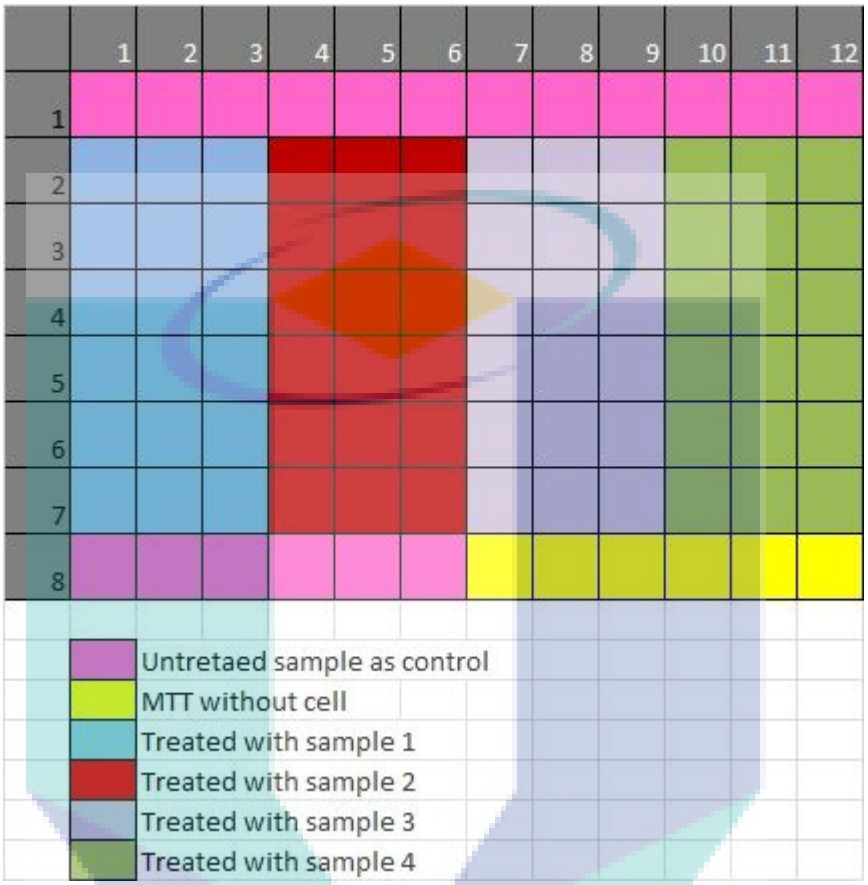


Figure D-1: 96-well plate during the cytotoxicity analysis with example of 4 different samples and triplicate run.

APPENDIX E

LIST OF PUBLICATIONS

1) Published in the **Journal of Life Sciences and Technologies (JOLST, ISSN: 2301-3672)** as one volume, reviewed by Ei Compendex and ISI Proceedings.

   Paper Tittle: Effect of Lectin from *Artocarpus Heterophyllus* Seed on Cancer Cell Lines

2) **4th International Conference on Chemical and Bioprocess Engineering (ICCBPE 2012)**

   Paper Tittle: Effect of Time in Extraction of Jacalin from *Artocarpus Heterophyllus* (Jackfruit) Seed

3) **2nd Malaysian International Conference on Trends in Bioprocess Engineering (MICOTribBE) 2012**

   Paper Tittle: Effect of Solid to Liquid Ratio in Extraction of Jacalin from Jackfruit Seed

APPENDIX F

PREPARATION OF JACKFRUIT SEED POWDER

Table F-1: List of jackfruit weighs

List of Jackfruit	Weigh of fresh fruit (kg)	Weigh of seed (g)	Weigh of dried chips (g)
Jackfruit 1	22	1023	405
Jackfruit 2	23	910	322
Jackfruit 3	24	752	264
Jackfruit 4	24	481	198
Jackfruit 5	20	574	214
Jackfruit 6	22	909	305
Jackfruit 7	18	757	285
Jackfruit 8	20	856	299
Total	173	6262	2292

Percentage average seed to fresh fruits :

= (weigh of seed / weigh of fruit) x100

= (6.26 kg / 173.00 kg) x 100

= 3.62 %

Percentage average dried chips seed :

= (weigh of dried chips / weigh of fresh seed) x100

= (2.29 kg / 6.26 kg) x 100

= 36.58 %

40 g weigh loss while grinding of chips into powder and nett weigh of seed powder collected was 2.25 kg.

APPENDIX G

RESULT OF PRETREATMENT EXPERIMENTS

Table G-1: Experimental design and results of soaking pretreatment at constant liquid to solid ratio 10 mL/g, temperature 4 ^{o}C and recovery time 24 hr.

Experiments	Soaking Time (hr)	Concentration (mg/mL)				Standard Deviation
		Run 1	Run 2	Run 3	Average	
A1	0	22.380	22.036	22.070	22.162	0.190
A2	1	25.154	26.405	25.289	25.616	0.687
A3	3	26.422	26.107	27.036	26.522	0.473
A4	6	29.807	30.060	27.839	29.236	1.216
A5	12	22.354	22.394	22.383	22.377	0.021
A6	24	22.036	22.291	22.326	22.217	0.158

APPENDIX H

RESULT OF CRUDE PROTEIN RECOVERY (OFAT)

Table H-1: Experimental design and results of crude protein recovery with parameter liquid to solid ratio (LSR). The experiment conducted at constant temperature 4°C and recovery time 3 hr.

Run Order	Standard Order	Factor 1 A: LSR (mL/g)	Concentration (mg/mL)				Standard Deviation
			Run 1	Run 2	Run 3	Average	
6	1	3	28.981	26.991	31.109	29.03	4.43
3	2	3	21.092	21.591	21.770	21.48	0.35
8	3	6	20.011	20.893	19.672	20.19	0.63
5	4	9	18.998	20.034	20.940	19.99	0.97
1	5	9	17.562	12.420	9.138	13.04	4.25
7	6	12	10.675	11.002	8.723	10.13	1.23
4	7	15	7.567	6.983	6.957	7.17	0.34
2	8	15	5.005	6.456	4.066	5.18	1.20

Table H-2: Experimental design and results of crude protein recovery with parameter temperature. The experiment conducted at constant liquid to solid ratio and recovery time 3 hr.

Run Order	Standard Order	Factor 3 B:Temperature (°C)	Concentration (mg/mL)				Standard Deviation
			Run 1	Run 2	Run 3	Average	
6	1	4	20.561	23.090	20.233	21.29	1.56
3	2	4	22.450	22.900	21.636	22.33	0.64
4	3	13	21.090	23.020	22.254	22.12	0.97
7	4	22	24.500	25.111	26.301	25.30	0.92
5	5	22	25.003	25.770	26.596	25.79	0.80
8	6	31	24.987	26.015	26.418	25.81	0.74
1	7	40	23.900	23.116	22.176	23.06	0.86
2	8	40	24.763	23.997	25.862	24.87	0.94

Table H-3: Experimental design and results of crude protein recovery with parameter recovery time. The experiment conducted at constant temperature 4 ^{o}C and liquid to solid ratio 30 mL/g.

Run Order	Standard Order	Factor 2 B:Time (hr)	Concentration (mg/mL)				Standard Deviation
			Run 1	Run 2	Run 3	Average	
6	1	0	17.986	19.003	19.345	18.78	0.71
3	2	0	16.987	17.450	16.729	17.06	0.37
8	3	3	25.671	29.320	29.976	28.32	2.32
5	4	6	32.895	34.562	32.080	33.18	1.27
1	5	6	33.786	34.872	30.666	33.11	2.18
7	6	9	29.439	34.675	33.019	32.38	2.68
4	7	12	25.678	25.342	25.296	25.44	0.21
2	8	12	26.867	26.567	27.101	26.85	0.27

APPENDIX I

RESULT OF OPTIMIZATION BY RSM

Table I-1: Experimental design and results RSM of crude protein recovery with three dependent variables, liquid to solid ratio (LSR), temperature and recovery time.

Std	Run	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)	Concentration (mg/mL)				Standard Deviation
					Run 1	Run 2	Run 3	Average	
20	1	25.00	31.00	5.00	44.829	45.191	44.902	44.974	0.191
35	2	30.00	28.00	6.00	41.937	42.298	41.792	42.009	0.261
42	3	30.00	34.00	4.00	45.697	45.914	45.046	45.553	0.452
43	4	30.00	28.00	4.00	38.248	37.525	38.538	38.104	0.521
12	5	35.00	25.00	5.00	48.445	48.518	48.807	48.590	0.191
9	6	25.00	25.00	5.00	41.141	39.839	40.273	40.418	0.663
21	7	25.00	31.00	5.00	42.804	42.154	43.672	42.877	0.762
16	8	35.00	31.00	3.00	58.425	55.026	61.607	58.353	3.291
4	9	35.00	25.00	3.00	54.158	52.567	55.966	54.231	1.701
29	10	40.00	28.00	4.00	34.416	34.271	34.343	34.343	0.072
25	11	20.00	28.00	4.00	34.705	33.620	33.837	34.054	0.574
24	12	35.00	31.00	5.00	34.633	34.416	34.633	34.560	0.125
46	13	30.00	28.00	4.00	34.705	35.139	34.488	34.777	0.331
47	14	30.00	28.00	4.00	39.116	39.333	39.116	39.189	0.125
26	15	20.00	28.00	4.00	33.693	33.909	33.909	33.837	0.125
30	16	40.00	28.00	4.00	34.850	34.994	34.488	34.777	0.261
45	17	30.00	28.00	4.00	39.116	39.695	38.321	39.044	0.690
19	18	25.00	31.00	5.00	42.371	42.588	41.720	42.226	0.452
8	19	25.00	25.00	5.00	54.664	54.881	57.051	55.532	1.320
6	20	35.00	25.00	3.00	39.623	40.346	38.899	39.623	0.723
23	21	35.00	31.00	5.00	27.690	30.077	24.870	27.546	2.606
39	22	30.00	22.00	4.00	50.181	46.131	47.071	47.794	2.120
48	23	30.00	28.00	4.00	40.997	41.141	40.635	40.924	0.261
10	24	35.00	25.00	5.00	52.784	53.652	52.784	53.073	0.501
13	25	25.00	31.00	3.00	54.231	54.158	54.303	54.231	0.072
11	26	35.00	25.00	5.00	41.864	44.178	39.116	41.720	2.534
2	27	25.00	25.00	3.00	36.296	34.850	34.922	35.356	0.815
33	28	30.00	28.00	2.00	40.997	41.864	42.298	41.720	0.663
3	29	25.00	25.00	3.00	43.021	42.804	42.371	42.732	0.331
32	30	30.00	28.00	2.00	44.034	44.178	43.672	43.962	0.261
28	31	40.00	28.00	4.00	32.535	33.186	32.102	32.608	0.546
27	32	20.00	28.00	4.00	33.476	33.837	32.897	33.403	0.474
7	33	25.00	25.00	5.00	42.660	43.455	43.166	43.094	0.403
31	34	30.00	28.00	2.00	41.213	41.575	40.635	41.141	0.474

Std	Run	Factor 1 A: LSR (mL/g)	Factor 2 B:Temperature (^{o}C)	Factor 3 C:Time (hr)	Concentration (mg/mL)				Standard Deviation
34	35	30.00	28.00	6.00	44.612	44.106	45.119	44.612	0.506
15	36	25.00	31.00	3.00	41.647	41.720	41.141	41.503	0.315
44	37	30.00	28.00	4.00	34.850	33.259	40.346	36.151	3.719
18	38	35.00	31.00	3.00	37.236	37.164	38.176	37.525	0.565
40	39	30.00	34.00	4.00	45.046	45.480	44.178	44.902	0.663
38	40	30.00	22.00	4.00	47.360	45.697	48.373	47.143	1.351
5	41	35.00	25.00	3.00	26.678	26.822	24.798	26.099	1.130
1	42	25.00	25.00	3.00	49.385	48.445	47.722	48.518	0.834
22	43	35.00	31.00	5.00	41.503	41.864	41.141	41.503	0.362
17	44	35.00	31.00	3.00	46.493	46.203	46.565	46.420	0.191
36	45	30.00	28.00	6.00	43.021	43.600	42.660	43.094	0.474
41	46	30.00	34.00	4.00	43.745	43.455	42.949	43.383	0.403
37	47	30.00	22.00	4.00	49.819	47.867	49.819	49.168	1.127
14	48	25.00	31.00	3.00	41.213	41.069	41.792	41.358	0.383

APPENDIX J

RESULT OF JACALIN EXTRACTION BY REVERSE MICELLE METHOD

Table J-1: Experimental design and results of jacalin extraction using reverse micelle for salt concentration variables study.

Experiment	Salt Concentration (M)	Concentration (mg/mL)				Standard Deviation
		Run 1	Run 2	Run 3	Average	
B1	0.1	1.1198	1.0708	1.1443	1.112	0.037
B2	0.2	1.7324	1.5854	1.6344	1.651	0.075
B3	0.5	1.3894	1.4139	1.4384	1.414	0.025
B4	1.0	1.2668	1.2914	1.3894	1.316	0.065
B5	1.5	0.8503	0.8993	0.8748	0.875	0.025
B6	2.0	0.7768	0.7523	0.8013	0.777	0.025
B7	2.5	0.7523	0.7523	0.6788	0.728	0.042

Table J-2: Experimental design and results of jacalin extraction using reverse micelle for pH value variables study

Experiment	Salt Concentration (M)	Concentration (mg/mL)				Standard Deviation
		Run 1	Run 2	Run 3	Average	
C1	5	0.7523	0.8748	0.8748	0.834	0.071
C2	6	1.6834	1.8304	1.8304	1.781	0.085
C3	7	1.3404	1.3894	1.3894	1.373	0.028
C4	8	0.8993	0.8258	0.8258	0.850	0.042
C5	9	0.7768	0.8748	0.8748	0.842	0.057
C6	10	0.9728	0.8993	0.8993	0.924	0.042
C7	11	0.7523	0.8258	0.8258	0.801	0.042
C8	12	0.6543	0.8258	0.8258	0.769	0.099

APPENDIX K

RESULT OF CYTOTOXICITY TOWARDS MCF7 AND H1299

Table K-1: Experimental design and results of cytotoxicity analysis on small lung carcinoma cell (H1299)

Experiment	Concentration (µg/mL)	Viability Cell (%)				Standard Deviation
		Run 1	Run 2	Run 3	Average	
Crude Protein						
G1	50.00	90	92	93	91.67	1.53
G2	100.00	89	87	91	89.00	2.00
Jacalin Extracted						
H1	50.00	78	80	81	79.67	1.53
H2	100.00	75	73	72	73.30	1.53
Jacalin Standard						
I1	50.00	60	62	64	62.00	1.63
I2	100.00	50	53	56	53.00	2.45

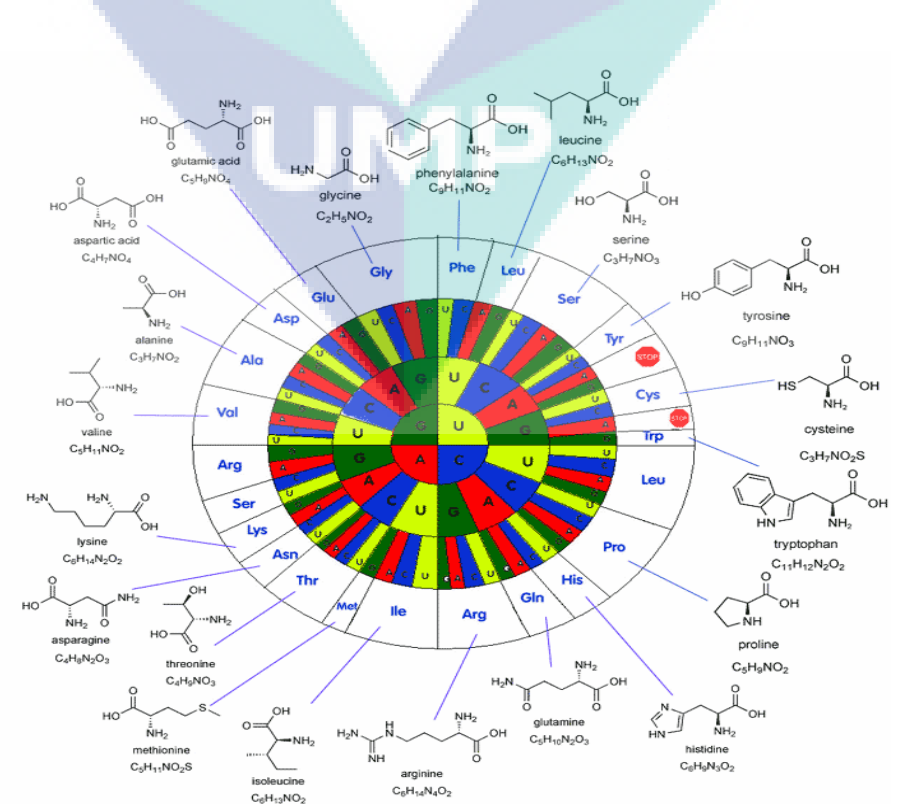
Table K-2: Experimental design and results of cytotoxicity analysis on human breast cancer cell (MCF7)

Experiment	Concentration (µg/mL)	Viability Cell (%)				Standard Deviation
		Run 1	Run 2	Run 3	Average	
Crude Protein						
D1	50.00	97	98	99	98.00	1.00
D2	100.00	89	87	85	87.00	2.00
D3	200.00	83	80	82	81.67	1.53
Jacalin Extracted						
E1	50.00	80	80	82	80.67	1.15
E2	100.00	62	65	64	63.67	1.53
E3	200.00	8	7	4	6.33	2.08
Jacalin Standard						
F1	50.00	75	76	78	76.33	1.53
F2	100.00	65	64	66	65.00	1.00
F3	200.00	8	4	4	5.33	2.31

APPENDIX L

AMINO ACID CODING

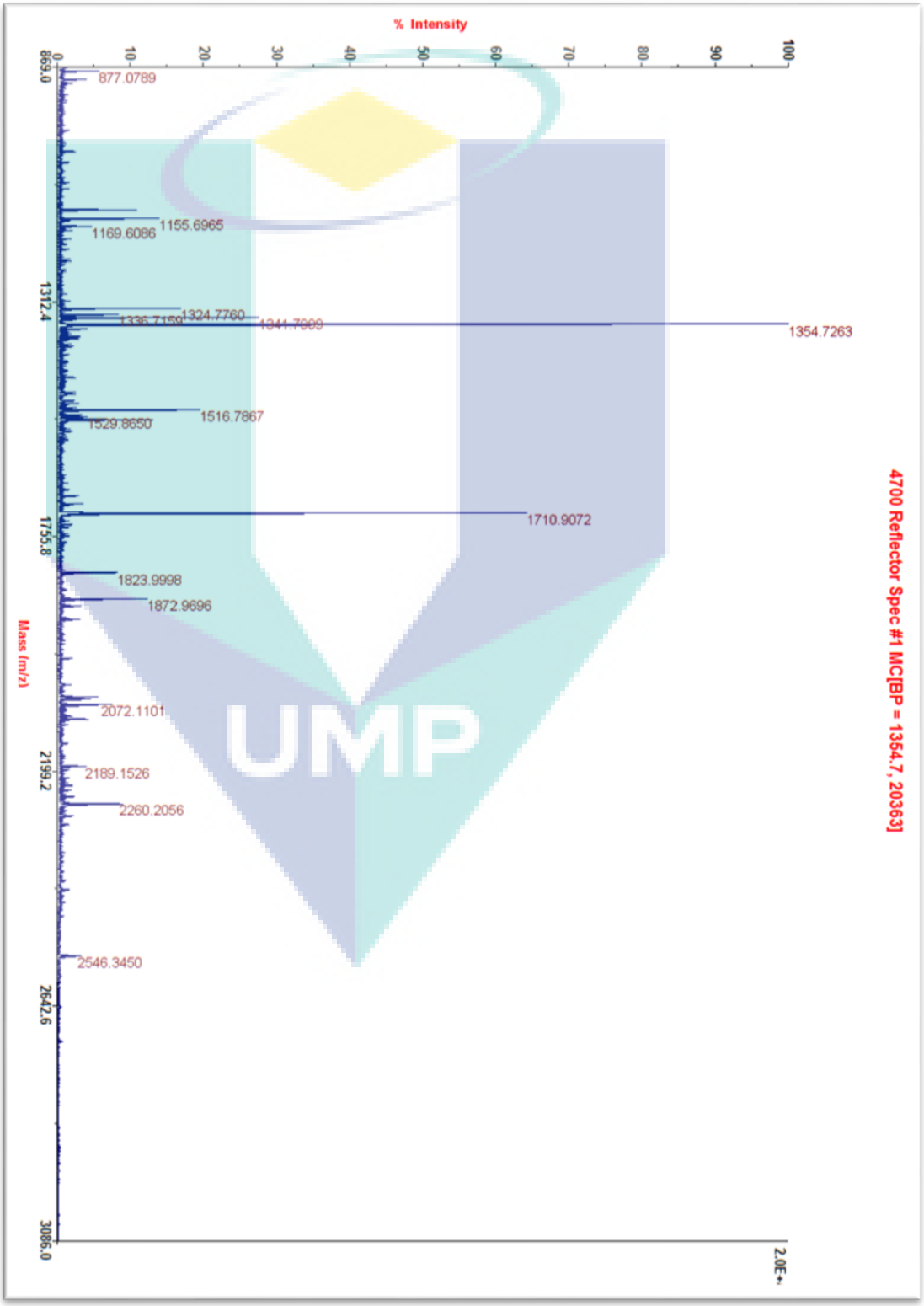
Amino Acid	Coding	
Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoluesine	Ile	I
Lysine	Lys	K
Leusine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Try	Y



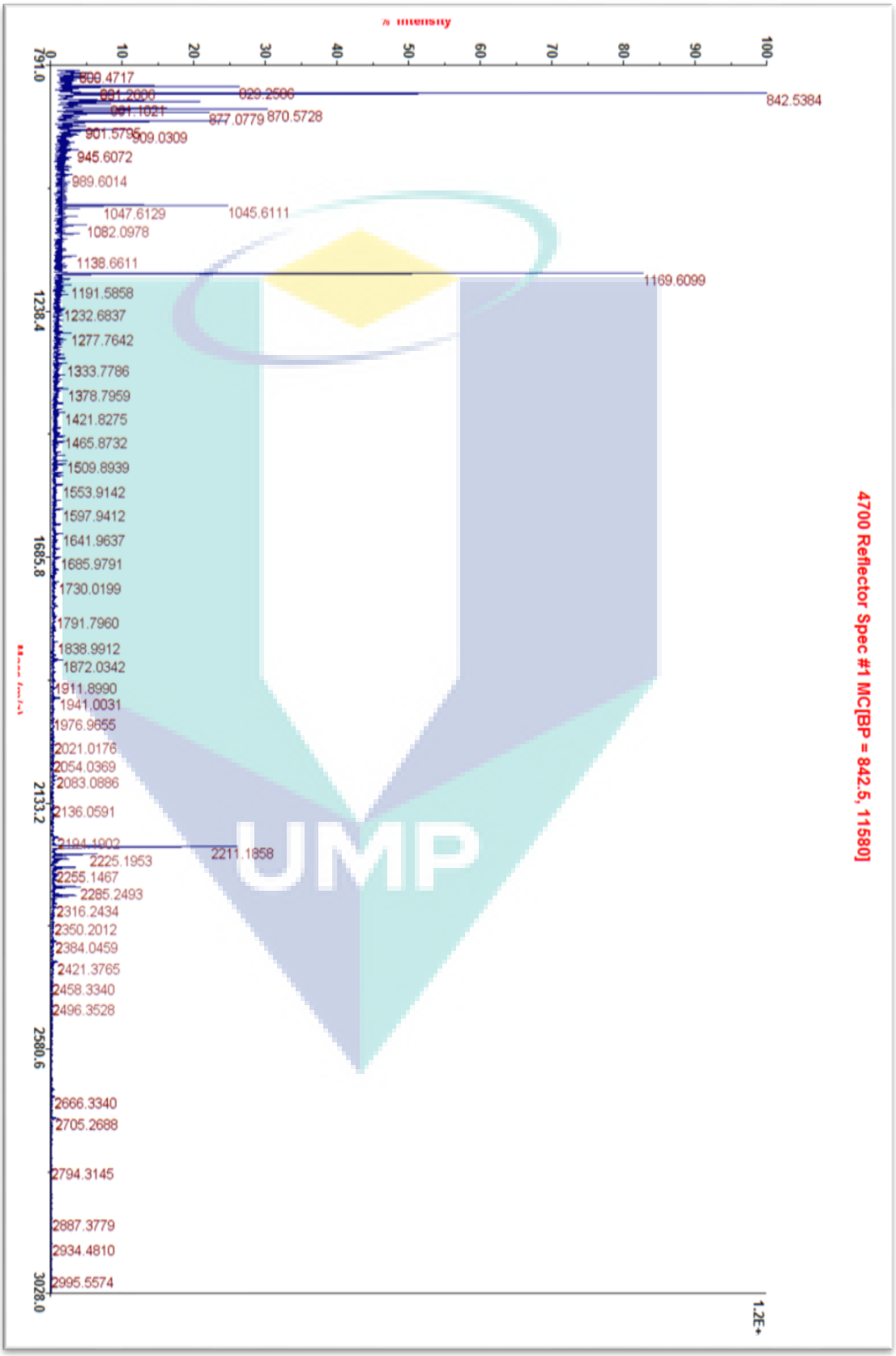
APPENDIX M

MALDI TOF SPECTRUM

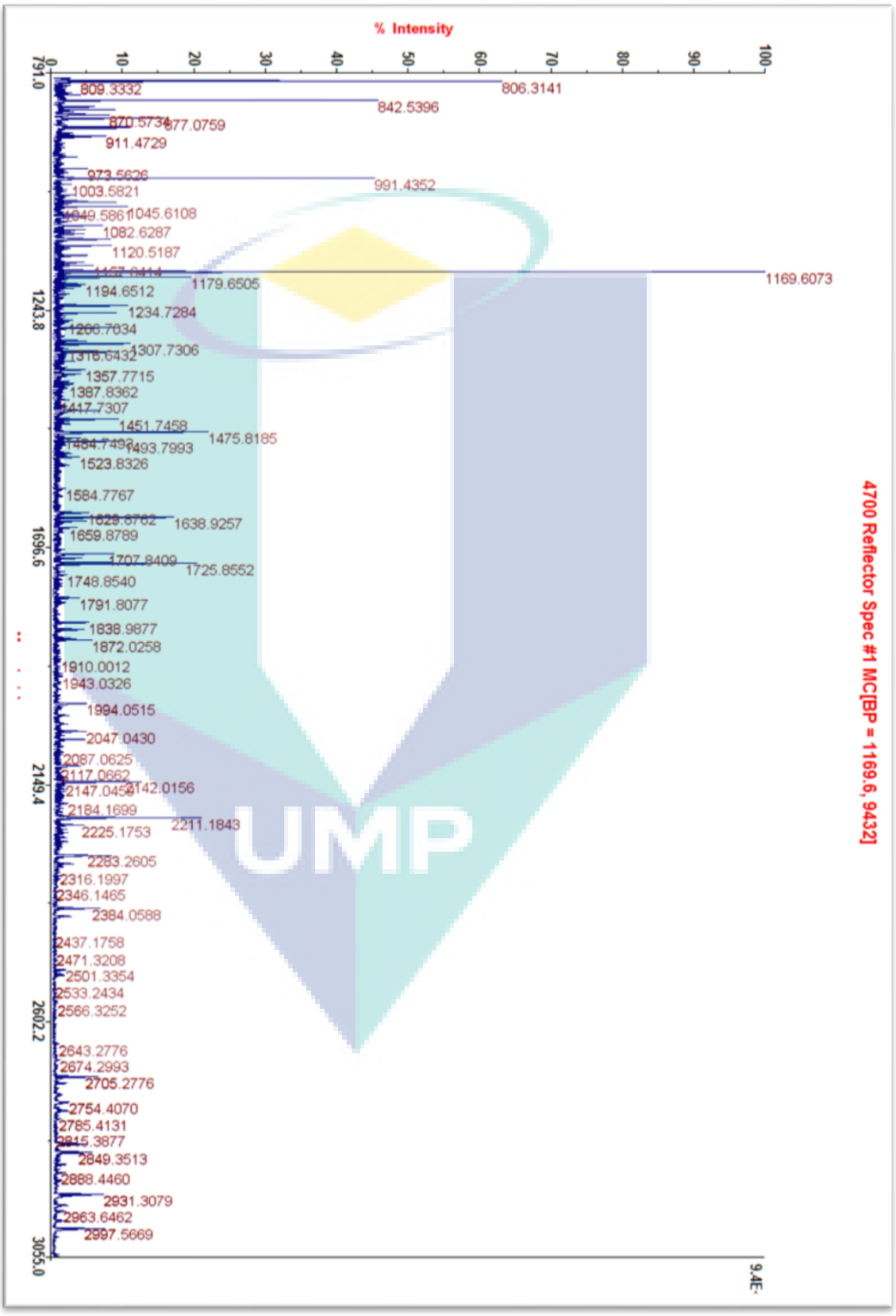
i) Crude Protein spectrum



ii) Jacalin extracted spectrum



iii) Jacalin standard spectrum



APPENDIX N

RESULT OF COMPOSITION OF JACKFRUIT SEED POWDER

(Nangka Madu Mastura)

TEST CERTIFICATE

Issued By: **Quality Manager**
Date of Issue: 13/03/2013
Serial Number: **E-13(040)**

MS ISO/IEC 17025
TESTING
SAMM NO. 224

Analysis Code & Name	Results	Units	Method*	Accredited (Y/N)
13BE133				
MADU				
Labeling				
Moisture	10.1	g/100g	D23A/M1M -1	Yes
Ash	3.2	g/100g	D23A/M2A-1	Yes
Crude protein	12.2	g/100g	D23A/M5P-1	Yes
Crude fat	0.0	g/100g	D23A/M3CFT-1	Yes
Crude fibre	3.0	g/100g	D23A/M4CFE-1	Yes
Carbohydrate	74.5	g/100g		No
Energy	347	kcal/100g		No
major & trace				
Calcium	59.4	mg/100g		No
Sodium	14.8	mg/100g		No
Magnesium	119.0	mg/100g		No
Potassium	1469.6	mg/100g		No
Iron	2.1	mg/100g		No
Copper	1.1	mg/100g		No
Zinc	1.5	mg/100g		No
Oil & Fats				
Free fatty acid	0.0	g/100g oil		No
Peroxide value	0.0	mEq/kg oil		No

Approved Signatory
NORFAIZA BT. ISMAIL
A/1907/2638/96/02
Research Officer
Food & Agricultural Analysis Laboratory Programme
Technical Services Centre
MARDI, Serdang

APPENDIX O

RESULT OF PROTEIN IDENTIFICATION

(MASCOT)