PRODUCTION OF EXTRACELLULAR PROTEASE ENZYME BY *ASPERGILLUS NIGER*

SITI KHAIRUNNISA BTE DAUD

Report submitted in fulfillment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering in Biotechnology

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2012

BORANG PENGESAHAN STATUS TESIS♦

JUDUL : PRODUCTION OF EXTRACELLULAR PROTEASE BY *ASPERGILLUS NIGER*

SESI PENGAJIAN : 2011/2012

Saya **SITI KHAIRUNNISA BTE DAUD**

(HURUF BESAR)

mengaku membenarkan tesis (PSM/~~Sarjana~~/~~Doktor Falsafah~~)* ini disimpan di Perpustakaan Universiti Malaysia Pahang dengan syarat-syarat kegunaan seperti berikut :

1. Tesis adalah hakmilik Universiti Malaysia Pahang
2. Perpustakaan Universiti Malaysia Pahang dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (√)

[ ] SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)

[ ] TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

[√] TIDAK TERHAD

Disahkan oleh:

(TANDATANGAN PENULIS) (TANDATANGAN PENYELIA)

Alamat Tetap: **22, JALAN LANGKAT 1,** SITI HATIJAH BINTI MORTAN

TAMAN INDAH, 83050 Nama Penyelia

BATU PAHAT, JOHOR

Tarikh : Tarikh:

CATATAN : * Potong yang tidak berkenaan.

Jika tesis ini **SULIT** atau **TERHAD**, sila lampirkan surat daripada pihak berkuasa/organisasiberkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai **SULIT** atau **TERHAD**.

Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau Lapuran Projek Sarjana Muda (PSM).

SUPERVISOR'S DECLARATION

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

Signature
Name of Supervisor: MISS SITI HATIJAH BINTI MORTAN
Position:
Date:

STUDENT'S DECLARATION

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

Signature
Name: SITI KHAIRUNNISA BTE DAUD
ID Number: KE08005
Date:

ACKNOWLEDGEMENTS

A very thankful to my beloved supervisor, Miss Siti Hatijah Binti Mortan, for her kindness and willingness in guiding, advising and continuously encouraging in order to finish up this report. The supervision and support that she gave was truly helpful in the progression of Undergraduate Research Project (URP). The co-operation is much indeed appreciated. Without her, this report was not able to be finished as expected.

These thanks also go to all my laboratory mates and members of the staff of Faculty of Chemical and Natural Resources Engineering, UMP who really helped me in many ways. Greatly thanks to research member group for their excellent cooperation, inspirations and supports during the study.

To my parents, thank you so much for the supports that really blow up my spirit and encourage me so well in this study. From money, sacrifice and everything, both of you have done so much for the sake of me. Thank you so much.

ABSTRACT

The research aimed to study the production of extracellular protease enzyme using potato peel extract as the additional carbon source for *Aspergillus niger*. Proteases are catalytically functioned to hydrolyze or breakdown the peptide bonds of proteins. Proteases are found to be used in many biotechnological processes and industrial applications such as in baking industry for gluten development, dairy industry as milk-clotting agents and pharmaceutical industries. Wastes from the agricultural and food industry gives out serious problem and as an action of initiative, those wastes can be used up and converted into value added materials as well as cost-effective substrates for fermentation of extracellular protease enzyme. Furthermore, in order to produce high yields of protease enzymes, the optimization of parameters is considered vital since it takes a long time and expensive to be optimized conventionally. This study was performed by using Response Surface Methodology (Central Composite Design). *Aspergillus niger* had been chosen as biomass while potato peel from the agricultural and food industry was used as additional substrate in this study. The potato peel will be grinded and blended with peel:water ratio of 1:3. The fermentation study will be take place in the shake flasks and several parameters were optimized for higher protease enzyme activity. Three factors were taken into considerations which were the pH of the fermentation medium (pH 3.5 – pH 7.5), the substrate concentration (20 g/l – 60 g/l) and the agitation speed (100 rpm – 300 rpm). From OFAT analysis, protease enzyme showed the optimum activity at pH 5.50, 40 g/l and 200 rpm with 1.23 U/ml, 1.57 U/ml and 1.38 U/ml, respectively while RSM results depicted that the optimum values of each parameter were 5.5 for pH, 40 g/l of substrates concentration and 200 rpm of agitation speed which gave out the optimum protease activity of 2.4563 U/ml. As the conclusion, RSM is the best tool used to identify the correlation between controlled independent factors and observed dependent responses and the utilization of waste as the fermentation substrates is highly acceptable due to its higher protease activity. For future study, it is recommended for an optimization of potato peel extracts concentration as the main carbon source, the application of genetic engineering in the enzyme production, further scale up protease production using a bioreactor and purification and toxicology studies on protease enzyme for further used by human, food and pharmaceutical industries.

ABSTRAK

Penyelidikan ini bertujuan untuk mengkaji penghasilan enzim protease di luar sel menggunakan ekstrak kulit kentang sebagai sumber karbon tambahan untuk *Aspergillus niger*. Protease berfungsi untuk menghidrolisis dan memutuskan ikatan-ikatan peptida yang terdapat pada protein. Ia banyak digunakan dalam proses-proses bioteknologi dan diaplikasikan dalam pelbagai industri seperti industri penaik sebagai peningkat gluten, industri tenusu sebagai agen penggumpal susu dan industri farmaseutikal. Peningkatan bahan buangan yang terhasil daripada industri pertanian dan industri makanan menjadi satu masalah besar dan sebagai langkah inisiatif, bahan-bahan buangan tersebut boleh digunakan dan ditukar menjadi sesuatu yang bernilai sebagai substratu berkos rendah dalam proses fermentasi untuk penghasilan enzim protease. Tambahan pula, pengoptimuman parameter-parameter tertentu sangat penting untuk meningkatkan penghasilan enzim protease. Pengoptimuman secara konvensional mengambil masa yang lama dan memerlukan kos yang tinggi. Kajian ini dijalankan dengan menggunakan Kaedah Tindak Balas Permukaan (Reka Bentuk Komposit Berpusat). *Aspergillus niger* telah dipilih sebagai biomas manakala kulit kentang dari industri pertanian dan industri makanan telah digunakan sebagai substratu dalam kajian ini. Kulit kentang dikisar dan dicampur dengan nisbah kulit kentang (1) kepada air (3). Kajian fermentasi ini dilakukan dalam kelalang goncang dan sesetengah parameter telah dikaji untuk penghasilan enzim protease yang optimum. Tiga parameter telah dikaji iaitu pH media fermentasi (pH 3.5 – pH 7.5), kepekatan substratu (20 g/l – 60 g/l) dan kelajuan pengadukan (100 rpm – 300 rpm). Merujuk kepada analisa OFAT, enzim protease menunjukkan aktiviti optimumnya berlaku pada pH 5.50, 40 g/l dan 200 rpm dengan 1.23 U/ml, 1.57 U/ml dan 1.38 U/ml, masing-masing manakala keputusan RSM menunjukkan nilai optimum untuk setiap parameter ialah pH 5.50, kepekatan substratu pada 40 g/l dan kelajuan pengadukan pada 200 rpm dengan aktiviti protease optimumnya pada 2.4563 U/ml. Kesimpulannya, RSM merupakan alat yang terbaik untuk mengenalpasti kaitan antara faktor bebas yang boleh dikawal dengan faktor bergantung yang diperhatikan dan penggunaan bahan buangan sebagai substratu bagi proses fermentasi adalah sangat digalakkan memandangkan lebih banyak enzim protease yang dihasilkan. Untuk kajian masa depan, adalah digalakkan untuk mengoptimumkan kepekatan ekstrak kulit kentang yang digunakan sebagai sumber karbon utama, penggunaan kejuruteraan genetik dalam penghasilan enzim, meningkatkan skala penghasilan enzim pada masa akan datang dengan menggunakan bioreaktor dan kajian tentang penulenan dan toksikologi enzim protease yang bakal digunakan oleh manusia, industri makanan dan farmaseutikal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

&	And
%	Percentage
β_0	Constant
$\beta_1, \beta_2, \beta_3$	Linear regression
$\beta_{11}, \beta_{22}, \beta_{33}$	Interaction regression
°C	Degree Celcius
µ	Micro
R^2	Determination of coefficient
$	Dolar
X_1	pH
X_2	Substrate concentration
X_3	Agitation speed
Y	Protease activity

LIST OF ABBREVIATIONS

3D	Three dimensional
ANOVA	Analysis of Variance
CCD	Central Composite Design
Corp.	Corporation
et al.	And others
etc	et cetera
$FeSO_4$	Ferum sulfate
HCl	Hydrochloric acid
g	Gram
g/l	Gram/Liter
GIA	Global Industry Analyst
GRAS	Generally Recognized As Safe
h	Hour
H_2O	Water
KH_2PO_4	Sodium dihydrogen phosphate
MARDI	*Malaysian Agricultural Research and Development Institute*
$MgSO_4$	Magnesium sulfate
ml	Mililiter
mM	Mili molar
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NH_2Cl	Ammonium chloride

nm	nanometer
OD	Optical density
OFAT	One-Factor-at-A-Time
PDA	Potato Dextrose Agar
rpm	Rotation per minute
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
SSF	Solid-State Fermentation
sp.	Species

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Protease is an enzyme that hydrolyzed peptide bonds (Salahuddin and Khan, 2008) and their usage in many industries is so much impressive. In dairy industry, protease is being used to coagulate the milk protein forming curds and ready to be used for cheese preparation. In food industry, proteases were used for improving the functional, nutritional and flavour properties in proteins especially in baking where it is used to degrade proteins in flour for biscuits, crackers and cookies. In pharmaceutical industry also, protease give a wide application such as in treatment of clotting disorder (Sumantha *et al.*, 2006). It has been used for treatment of clotting disorder in pharmaceutical industry while in detergent industry, used for protein stain removal. For leather industry, it usage is for unhearing and bating. According to Oyeleke *et al.* (2010), protease can easily isolate from various sources as well as plants, animals and microbial via fermentation process.

Enzyme production is a good value added to agro-industrial residues since they can be used in the production of enzymes by bioprocesses (Paranthaman *et al.*, 2009). This issue was related to **"**From waste to wealth" concept which is an idea that had been practiced nowadays by almost all people in the world. This statement gives an idea of using unwanted materials that can be recycled or becoming alternatives resources for other processes. The unwanted materials will basically refer to the wastes. An example of wastes come from agricultural and food industry because there are most abundant of agro-industrial residues on our Earth that actually possess such good potential as renewable

resources (Nigam and Pandey, 2009). The utilization of wastes as substrate in the industrial enzyme production has made the fermentation process of industrial enzymes economically feasible.

The aim of this study is to investigate the production of extracellular protease enzymes by *Aspergillus niger* using potato peel extracts as substrate and to optimize several fermentation parameters for optimum enzyme activity.

1.2 PROBLEM STATEMENT

Many researches has been done for developing processes to produce extracellular protease enzyme that involves various additional process for improvement in terms of economical, qualitative, quantitative, and process performance factors. The screening of microorganism, the preparation of the substrate from various sources and the selection of range of several parameters are examples of improved process being done.

Around 40% for the whole cost of production is the substrates cost (El Enshasy *et al.*, 2008). Thus, the availability of an inexpensive raw material; potato peel extracts which will be used in this research is essential if this fermentation is to become economically viable.

In fermentation, there is variety of parameters that could affect the process performance. Fermentation is known to be dependent on temperature, pH, rate of aeration, substrate concentration etc so, that is why optimization steps need to be done in order to maximize the production rate of protease enzymes (El Enshasy *et al.*, 2008). The parameters need to be controlled to provide the perfect fermentation conditions for the fermentation process.

1.3 OBJECTIVE

The objective of this research is to study the production of extracellular protease enzyme using potato peel extracts as the additional substrates for *Aspergillus niger*.

1.4 SCOPE OF THE STUDY

To achieve the objective, few scopes have been identified in this research:

a) To study the production of extracellular protease enzyme in submerged fermentation using potato peel extracts as the additional substrate for *Aspergillus niger*.
b) To study the effects of pH (pH 3.5, 4.5, 5.5, 6.5 and 7.5) to the extracellular protease production.
c) To study the effects of substrate concentration (20, 30, 40, 50 and 60 g/l) to the extracellular protease production.
d) To study the effects of agitation speed (100, 150, 200, 250 and 300 rpm) to the extracellular protease production.
e) To apply Response Surface Methodology (RSM) in designing the experimental work.

1.5 RATIONAL AND SIGNIFICANCE

The aim of this study was to explore for new beneficial sources of substrate from wastes of agricultural and food industry so that the cost of growth factors can be lower down. This study proposed in minimizing the waste problems in the industry and estimating the optimum parameters values for higher yield of extracellular protease production by using potato peel extracts as the substrate to *Aspergillus niger*. The application of Response Surface Methodology (RSM) was a step of development in today's technology and this study was to expose others with new born technology. Perhaps with the application of RSM, experimental works will be much easier, less time consuming and feasibly inexpensive. The experimental stages done and obtained from the present study may be review by other future researchers for further process development in this field.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This part included all the collected and well organised information related to the study from variety of literature being reviewed from journals and other related sources.

2.2 INDUSTRIAL ENZYMES

Smythe (1950) defined enzymes as organic catalysts which are produced by living cells and they are responsible in catalyzing the chemical reactions of process life of the cell. There are many different kinds of enzymes and their existence is actually undetermined since only few known enzymes had been discovered. Some people proposed that all proteins are enzymes.

Global Industry Analyst, Inc. (GIA) forecasting the global market for industrial enzymes by 2015 is approximately to be US$3.74 billion. This is due to new developed enzyme technologies that enhance the cost efficiencies and productivity, customers' favour in substituting petrochemical products with organic products and lastly because of the high demand from textile, detergent, cosmetic and pharmaceuticals manufacturers (Global Industry Analysts, 2011). The common enzymes are lipases, carbohydrases, proteases and many more. The role of industrial enzyme had also been studied by Kirk *et al*. (2002) and is showed in the Table 2.1.

Table 2.1: Role of industrial enzymes

Industry	Enzyme class	Application
Detergent (laundry and dish washing)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour clarification, anti-redeposition (cotton)
	Mannonase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerise	Glucose to fructose conversion
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavour
	Lipase	Cheese flavour
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths

Table 2.1: Continued

Industry	Enzyme class	Application
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	β-glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-glucanase	Mashing
	Laccase	Clarification (juice), flavour (beer)
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling

Source: Kirk *et al.* (2002)

One of the largest product segments in global industrial enzyme market is proteases (Rao *et al*., 1998; Global Industry Analysts, 2011). Proteases monopolized around 60% of the world's industrial enzyme market as they are found to be used in many biotechnological processes and industrial applications (Rao *et al*., 1998; El Enshasy *et al.*, 2008) such as in baking industry for gluten development, dairy industry as milk-clotting agents (Sumantha *et al.*, 2006) and pharmaceutical industries (Sambamurthy and Kar, 2006) and this is clearly seen in Figure 2.1.

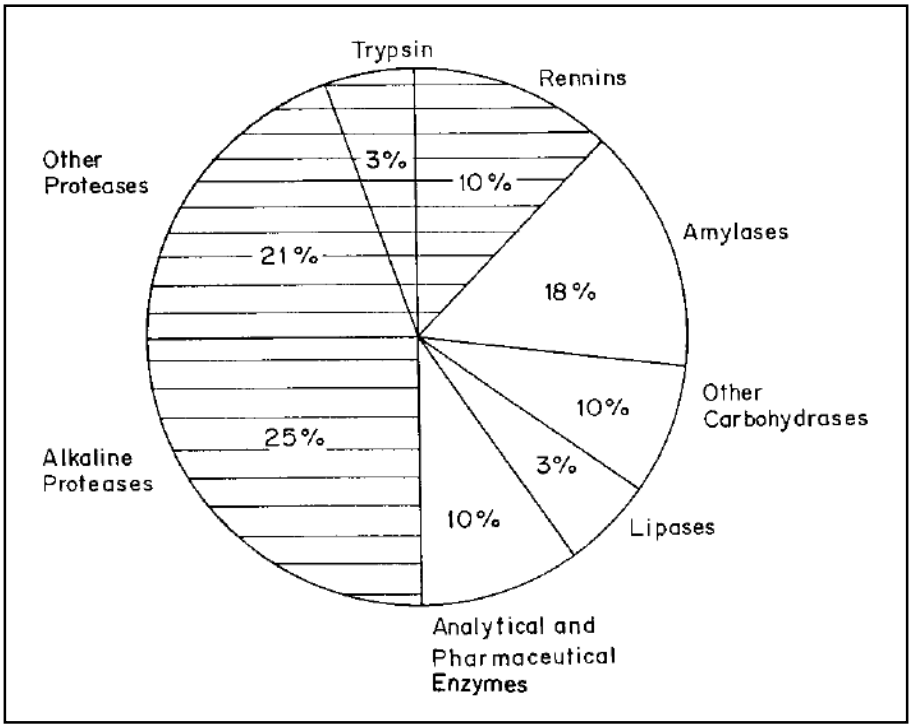


Figure 2.1: Distribution of enzymes sale

Source: Rao *et al*., 1998.

The Figure 2.1 describes the sale distribution of enzymes. The shaded area represents the sale of proteases enzyme and it shows that half of the sale is monopolized by protease. This figure really proved that proteases are important enzyme used in the world.

Since proteases were found to have such numerous applications in the industries, they were commercially produced nowadays.

2.3 PROTEASE

Proteases, also known as proteolytic enzymes or proteinases, belong to a group of enzymes whose catalytically function to hydrolyze or breakdown the peptide bonds of proteins and they can either be limited proteolysis which break specific peptide bonds or unlimited proteolysis which break down a complete polypeptide chain to amino chain residues (Salahuddin and Khan, 2008).

These enzymes can be found from various sources such as plants, animals and microorganisms (Rao *et al*., 1998; Ikram-Ul-Haq *et al.*, 2006). Protease can be classified according to three major criteria which are the type of reaction catalyst, chemical nature of the catalytic site and evolutionary relationship with reference to structure. Rao *et al.* (1998) studied further about microbial proteases. There are two major fungi that responsible in the production of protease which is filamentous fungi and yeast and can be further classified to acidic protease, alkaline protease, serine protease and metalloprotease (filamentous fungi)/other protease (yeast).

Mostly they were produced by fungi, bacteria and viruses (microbial proteases) (Rao *et al*., 1998; Ikram-Ul-Haq *et al.*, 2006) because of the inability of plants and animals proteases to satisfy the world demands (Rao *et al*., 1998; Kumar *et al.*, 2008). Adinarayana and Ellaiah (2002) also reported that two third of industrial proteases are microbial proteases. This is due to their broad biochemical diversity and susceptibility to genetic manipulation (Sandhya *et al.*, 2005) and also because of the characteristics of microbial proteases that can satisfy the need in biotechnological application (Kumar *et al.*, 2008). Table 2.2 shows the potential producers of proteases according to its types (Rao *et al*., 1998).

Table 2.2: Fungal proteases producers

Fungi	Type of proteases	Fungi species
Filamentous fungi	Acid proteases	a) *Mucor* b) *Rhizopus* c) *Aspergillus*
	Alkaline proteases	a) *Aspergillus* b) *Acremonium* c) *Fusarium*
	Serine proteases	a) *Tritirachium*
	Metalloproteases	a) *Aspergillus*
Yeast	Acid proteases	a) *Saccharomycopsis* b) *Saccharomyces* c) *Candida albicans, Candida tropicalis* d) *Yarrowia lipolytica*
	Alkaline proteases	a) *Yarrowia lipolytica*
	Serine proteases	a) *Kluyveromyces* b) *Saccharomyces cerevisiae*
	Other proteases	a) *Saccharomyces cerevisiae*

Source: Rao *et al*. (1998)

Almost all industries have long used bacterial proteases but the preparation for obtaining enzymes that free from microbes is quite costly (Andrade *et al.*, 2002). Due to this issue, fungal proteases have increase in their industrial demand since they offer several advantages compared to bacterial proteases.

Sandhya *et al.* (2005) and Murthy and Naidu (2010) stated that fungi were safe in producing enzymes because they were known as GRAS (Generally Recognized As Safe) strains and referring to Food and Drug Administration (2001), proteases from *Aspergillus niger* and *Aspergillus oryzae* origins were declared to be GRAS, meaning that they are safe to be consumed by human. In addition, fungal protease was produced extracellular that make ease to be recovered from the fermentation broth (Sandhya *et al.*, 2005; Murthy and Naidu, 2010). Moreover, the generated mycelium from the fermentation system can be easily tossed by simple filtration (Andrade *et al.*, 2002; Murthy and Naidu, 2010) and fungi

can also easily grown on inexpensive substrates (Murthy and Naidu, 2010; Benazir *et al.*, 2011). Besides, these microorganisms are also capable in consuming organic materials in wastes for their both carbon and energy sources in order to grow (Mahmood *et al.*, 1998).

Proteases can be produced either intracellularly or extracellularly. Mostly microbial proteases were produced extracellularly which means the proteases abundantly found within the production medium. Andrade *et al.* (2002) said that extracellular enzymes capable to digest insoluble nutrient materials such as cellulose, protein and starch, and the nutrients from digested products are then transported into the cell to be used as for growth.

2.4 *ASPERGILLUS NIGER*

Aspergillus niger is one of the known fungi that have the potential in producing extracellular protease enzymes due to its cosmopolitan and ubiquitous nature (Benazir *et al.*, 2011). The scientific classification of *Aspergillus niger* is described by Table 2.3.

Table 2.3: Taxonomy of *Aspergillus niger*

Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*Aspergillus niger*

Source: (Universal Protein Resources, 2011)

Aspergillus niger is a filamentous fungus that give an important role in biotechnology. *Aspergillus niger* does have spores and reproduces asexually meaning that it can produce its offspring individually. Purwanto *et al.* (2009) discovered the morphology of *Aspergillus niger* using Scanning Electron Microscope (SEM) while Hoffmann (2010) identified the SEM of the asexual reproduction of *Aspergillus niger*.

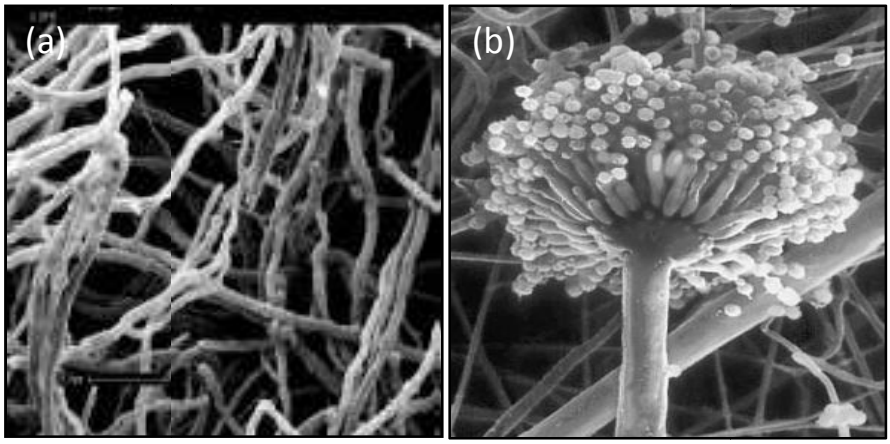


Figure 2.2: (a) Filamentous structure of *Aspergillus niger*
(b) Asexual reproduction of *Aspergillus niger*

Sources: (a) Purwanto *et al.* (2009)
(b) Hoffmann (2010)

According to Ikram-Ul-Haq *et al.* (2006), filamentous fungi do have potential to grow under varying environmental conditions such as fermentation time, pH, temperature and utilizing various sources of substrate as nutrients. Several fungi have been reported to produce proteases such as *Aspergillus niger* (Paranthaman *et al.*, 2009), *Aspergillus oryzae* (Murthy and Naidu, 2010), *Aspergillus fischeri* (Saravanakumar *et al.*, 2010), *Penicillium chrysogenum* (Ikram-Ul-Haq *et al.*, 2006) and *Mucor circinelloides* (Andrade *et al.*, 2002).

2.5 PRODUCTION OF PROTEASES

Microbial proteases have been commercially produced by fermentation process either in solid-state fermentation (SSF) or submerged fermentation (SmF). In large scale fermentation, SSF is not often used due to severe engineering problems and it was suggested to apply the submerged fermentation process as it possesses few advantages (Gregori *et al.*, 2007). Submerged fermentation was said to provide more uniform and reproducible biomass and also requires uncomplicated downstream processing.

Some researcher also discovered other method that was capable in producing high yield of protease enzymes. Samarntarn *et al.* (1999) used genetically engineered *Aspergillus oryzae* U1521 that enabled to produce five times more proteases yield than its parental strain since it contains multiple copies of protease gene while Dunne *et al.* (1997) had successfully forced *Stenotrophomonas maltophilia* W18 which was isolated from the rhizosphere of sugar beet to secrete extracellular protease activity for the purpose of biological control of *Pythium ultimum*. Unfortunately, the application of genetic engineered technique was still lacking among the researchers and most of them focused more in using the common fermentation technique either in submerged or solid state forms.

With regard to proteases synthesis in the microorganism, Saravanakumar *et al.* (2010) discovered that medium composition and some physical factors such as the pH, fermentation period, temperature etc have influenced the extracellular protease production. El Enshasy *et al.* (2008) decided that the improvement of yields of proteases and the optimization of the fermentation medium and production conditions need to be considered as to develop economically feasible technology. That was why many parameters were studied in former researches for examples, the pH, temperature, incubation time, different carbon and nitrogen sources, substrate concentrations and inoculums sizes. Better fermentation conditions will enhance higher production of proteases.

There will be many variables to be studied in every fermentation process. Studying one parameter at a time while holding the other parameters constant is the optimization

process that is done classically will normally takes such a long period of time and quite costly (Saravanakumar *et al.*, 2010). It also does not consider the effect of interaction of various parameters (Adinarayana and Ellaiah, 2002). Response Surface Methodology (RSM) is known as a useful model for studying factors that affect the responses by varying them simultaneously without much number of experiments to be carried out (Adinarayana and Ellaiah, 2002). With this, optimization procedure will be much easier to carry out.

2.5.1 Effect of pH on Protease Production

The pH is the measure of acidity or alkalinity of an aqueous solution and it has been proved strongly to affect the production of protease in the process of fermentation. It influences most of the enzymatic processes and the transport process of diverse of components across the cell membrane (Sandhya *et al.*, 2005; Paranthaman *et al.*, 2009; Murthy and Naidu, 2010).

The pH is related to the amount of hydrogen (H^+) concentration and this H^+ will give effect to the growth of the microorganisms. At neutral pH, it can act as the substrate that might be used by the cells in order to growth but in acidic and alkaline conditions, it can become inhibitor to the cell growth and this will affect the enzyme production by the cells. Natarajan and Rajendran (2009) also said that variety of pH values may also lead to the changes of protein structure meaning that maybe at certain pH the protease might be denatured once it is secreted to the outside of the cells or it can be said that the enzyme is already inactive and not stabile at that pH.

Murthy and Naidu (2010) found out that the increase in pH can cause the decrease of protease activity. In addition, different microorganisms might have different kinds of metabolic process and due to that the enzyme activity might also be affected. Coral *et al.* (2002) reported that fungal proteases are active at neutral pH and alkaline pH around pH 7 to pH 11 but Chakrabarthi *et al.* (2000) proved that *Aspergillus terreus* managed to produce protease between pH 5.5 and pH 9.5.

2.5.2 Effect of Substrate Concentration on Protease Production

All microorganisms need carbon sources in order to live as it is the food for them. According to Adinarayana *et al.* (2003), the carbon sources are the utmost nutritional factors that influenced the protease production.

Logically, if the microorganisms are supplied with adequate amount of nutrients, they will grow effectively meaning that they got all the nutrients needed in order to grow and product production will be maximized. If the microorganisms grow in lack of nutrients, their growth may be retarded and this will probably affect the product formation within the fermentation process.

Other metabolites or maybe the main products itself can repressed the product formation and it said that commonly the product production will decrease if the substrate concentration increase (Escobar and Barnett, 1993). When the substrate concentration is too high, it may also prohibit the enzyme activity (Dekleva *et al.*, 1985). Andrade *et al.* (2002) showed that protease activity was at its maximum when 40 g/l of substrate was used.

2.5.3 Effect of Agitation Speed on Protease Production

Agitation speed is one of the physical factors the influenced the fermentation process and at the same time give effect to the production of the product by the microorganisms. Several researchers did study the effect of agitation speed towards the protease production.

In the fermentation of aerobic culture, the oxygen really affects the production of product. It is because the metabolic pathway had been affected and the changes in metabolic fluxes occurred (Calik *et al.*, 1998). According to Ducros *et al.* (2009), the respiration rate of the aerobic culture is dependent to the dissolved oxygen if the dissolved oxygen reaches below its critical level. This is due to physiological alteration in cell metabolism (Hwang *et al.*, 1991). In order to avoid the reduce in cell growth and product

formation, it is necessary to make sure the agitation speed supplied for the fermentation is adequate enough since the agitation enhances the dispersion of air in the fermentation medium which makes it capable in maintaining the dissolved oxygen level (Ducros *et al.*, 2009; Singh *et al.*, 2011), equilibrate the temperature and the pH (Ducros *et al.*, 2009) and also improves nutrient transfer rate (Ducros *et al.*, 2009; Kamath *et al.*, 2010). As the matter of fact, high aeration rate causes by the agitation will improve enzyme synthesis.

Unfortunately, optimum enzyme production influences by diverse agitation ranges. Sepahy and Jabalameli (2011) suggested that agitation speed at 110 rpm and 130 rpm gave lower protease activity while some other researchers reported the optimum agitation speed for protease production from different isolates were at a range of 150 – 300 rpm (Banerjee *et al.*, 1999; Joo *et al.*, 2002; Kanekar *et al.*, 2002). Aerobic culture unable to grow in anaerobic condition so due to lack of aeration and nutrients uptake affected by the agitation, the cells cannot grow well but the same thing will also happened if the speed of agitation was too high. High speed will damage the microorganisms and its morphology due to shear stress and will also probably become the factor of low enzymatic activity (Ducros *et al.*, 2009; Sepahy and Jabalameli, 2011; Singh *et al.*, 2011).

2.6 UTILIZATION OF WASTES AS SUBSTRATE

Fermentation process needs nutrient medium that must satisfy the elemental requirements for the cell biomass, product formation and energy. The nutrients consist of macronutrients such as carbon sources, nitrogen sources and micronutrients such as trace elements and vitamins. According to El Enshasy *et al.* (2008), 40% of the production cost of large scale industrial enzyme is based on the cost of substrate so, it is important to use cost-effective substrate in the production process.

Normally most research used alternative sources of substrate to substitute the carbon and nitrogen sources in the fermentation process. There are many relevant sources of substrate to be used in the fermentation and the utilization of these alternative sources is hopefully promising for cheaper substrate cost but with efficient enzyme production especially in large scale production.

Agricultural, food and drinks industrial wastes are becoming abundant (Mahmood *et al.,* 1998) and the wastes disposal is problematic (Gregori *et al.,* 2007). Due to the pollution cause by this issue, Sarkar *et* al. (2011) had come out with biological treatment of these wastes via microbial degradation and it was the efficient method to be used in the future. By the way, these organic materials are composed of carbohydrate, amino acids, peptides and proteins, volatile acids, fatty acids and esters that are known to be biodegradable (Sarkar *et* al., 2011) while Mahmood *et al.* (1998) also discovered that the wastes of orange and potato peel extracts do have significant amount of carbohydrates except cellulose and they are readily to be used by the microorganisms while the microorganisms are said to be capable to utilize the organic matter in wastes as sources of either energy or carbon for their growth. Due to the above reasons, nowadays, the usage of agricultural, food and drinks wastes as fermentation substrates is highly increasing as it promotes lower cost requirement.

Recent works have studied a variety of wastes as carbon and nitrogen sources in the production of proteases such as orange and potato peel extracts (Mahmood *et al.,* 1998),

spent brewing grain, coconut oil cake, palm kernel cake, sesame oil cake, jackfruit seed powder and olive oil cake (Sandhya *et al*., 2005), rice husk (Sandhya *et al*., 2005; Ahmed *et al.*, 2010), rice bran (Sandhya *et al*., 2005; Ahmed *et al.*, 2010; Benazir *et al*., 2011), wheat bran (Sandhya *et al*., 2005; Kumar *et al*., 2008; Ahmed *et al.*, 2010; Benazir *et al*., 2011), groundnut cake (Kumar *et al*., 2008), varieties of rice broken (Paranthaman *et al.*, 2009), sunflower meal, soybean meal, cotton seed meal, rice polish (Ahmed *et al.*, 2010), coffee by-products (coffee pulp, coffee cherry husk, coffee parchment husk, silver skin, coffee spend wastes) (Murthy and Naidu, 2010), coconut bran, gingely oil bran, ground oil cake and black gram bran (Benazir *et al*., 2011). All the mentioned researches that utilized wastes as substrates in their studies discovered that although with the usage of cheap and easily available substrates but it really proved to efficiently produce enzymes.

The utilization of wastes in the fermentation of other several enzymes had also been reported. Gombert *et al.* (1999) has successfully produced lipase with the use of oil industrial waste (babassu oil cake) as substrate while Sidkey *et al.* (2010) able to utilize enviro-agro-industrial waste from food and drinks industry in the production of α-amylase. Ten different agroindustrial wastes were also reported in producing glucoamylase (Zambare, 2010).

According to Mahmood *et al.* (1998), the usage of agricultural wastes in developing countries are underutilized and if used but only in the production of single cell protein. On the contrary, in developed countries, these wastes are really useful in fermentation but still rarely used in enzymes production. The results from the studied recommended that these wastes can be used for better advantage. The common agricultural wastes are rice husk, coffee pulp and also potato peel.

According to Arapoglou *et al.* (2009) in the International Conference on Environmental Science and Technology entitled “Alternative Ways for Potato Industries Waste Utilization” in Greece, it was suggested to utilize wastes from the potato industries to be used for something beneficial since the problem due to this issue is of great concern. In order to overcome this problem, an environmental friendly solution is still under

investigation. In potato industry, potato starch waste and potato peel waste are the major wastes to be produced and about half of the potato industry waste production is monopolized by potato skin. In addition, potato peels contain sufficient amount of starch, cellulose, hemicellulose and fermentable sugars.

They have been used in the previous study and were approved good to serve as substrate in the production of extracellular protease enzymes (Mahmood *et al.*, 1998; Arapoglou *et al.*, 2009) and Table 2.4 shows the findings in a research that utilized potato and orange peel extracts as substrates.

Table 2.4: Effect of type of substrates on the production of extracellular proteases

Substrate	**Protease Activity (Units/ml)**	
	Alkaline protease	**Neutral protease**
Orange filtrate	7.5	20.0
Potato filtrate	11.0	33.0
Orange:Potato (1:1)	11.0	17.0
Glucose	4.0	7.5

Source: Mahmood *et al.* (1998)

From the table above, waste filtrates had been proved to potentially secrete optimum amount of protease compared to the utilization of glucose as substrate and potato peel extract presented the highest protease activity among the others. This issue is promising for industrial application (Arapoglou *et al.*, 2009).

2.7 OPTIMIZATION OF PROTEASE PRODUCTION

Nowadays, researchers are more focused in optimizing the process parameters so that high yield of protease can be achieved. Optimization is classically done by applying one-factor-at-a-time (OFAT) method where it is known to be very time-consuming method. There are several parameters are going to be optimized but in this method other parameters needs to be constant while studying a parameter at a time.

Fermentation is really affected by various factors such as pH, temperature, agitation speed, type of carbon and nitrogen sources and many others. In optimization, the best value for every studied factor is to be determined for its highest production of protease. For example, the effect of temperature to the fermentation process has been studied so that the exact temperature at which the protease yield is relatively high can be determined but by implementing OFAT method, each factor can only be studied alone since the other factors need to maintain constant. This method used to consume a long period of time if many parameters have to be studied. Furthermore, the effect of interaction between parameters cannot be observed in this way. The results obtained from OFAT can be further studied for the interaction between two or several factors.

Table 2.5 shows the optimization of parameters done by previous researcher. The research studied different parameters and came out with different outcomes (maximum protease activity). From the study, the researcher manage to identify at what range of parameters will actually result in high protease activity.

Unfortunately, the analysis for this kind of results is hard to be done. Therefore, Response Surface Methodology (RSM) is being introduced to facilitate in statistical analysis. In addition, RSM also helps in designing the experiment so that the experiment will cover all the range of parameters.

Table 2.5: Optimization of several parameters with the optimum protease activity

Species	State	Studied parameter	Value	Optimum protease activity
Aspergillus flavus	SmF	Incubation time	144 h	0.96 U/ml
		Temperature	30 °C	0.46 U/ml
		pH	8.0	0.74 U/ml
Aspergillus fumigatus		Incubation time	144 h	0.84 U/ml
		Temperature	30 °C	0.43 U/ml
		pH	5.0	0.70 U/ml

SmF submerged fermentation

Source: Oyeleke *et al.* (2010)

2.8 RESPONSE SURFACE METHODOLOGY

Optimization is important in order to improve the performance of the systems and to maximize the productivity of the process without increasing the cost (Bas and Boyaci, 2007). There will be many variables to be studied in every fermentation process. Studying one parameter at a time while holding the other parameters constant is the optimization process that is done classically and this will normally takes such a long period of time and quite costly, when large number of variables are evaluated (Saravanakumar *et al.*, 2010). This technique is called one-factor-at-a-time (Bas and Boyaci, 2007). It also does not consider the effect of interaction of various parameters (Adinarayana and Ellaiah, 2002; Bas and Boyaci, 2007) and it does not represent the complete effects of the parameters (Bas and Boyaci, 2007) so that is why Response Surface Methodology (RSM) is being introduced and recommended for optimization purpose.

Annadurai and Sheeja (1998) described RSM as an empirical modelization method used in the evaluation of the relationship of a set of controlled experimental factors and observed responses. RSM is a useful tool for studying factors that affect the responses by varying them simultaneously and it can also be used to study the relationships between one or more factors (independent variables) and responses (dependant variables) (Adinarayana

and Ellaiah, 2002). RSM can be applied in both chemical and biochemical processes (Bas and Boyaci, 2007).

Bas and Boyaci (2007) said that while using RSM, the optimization process should undergo three vital stages. The first stage is the preliminary study for estimating the independent parameters to be carry out. The second stage is choosing the experimental design and also predicting and verifying the model equation. The last stage is used to obtain the response surface plot and contour plot of the response and finally determining the optimum values.

The experimental design is required to allow the quadratic model to be fitted and minimized lack of fit of the model but still having enough degrees of freedom for pure error determination (Khuri, 2006). An efficient analysis should satisfy the following criteria. First, the second order model should efficiently fit. Second, the lack of fit of the model is also checked. Then, the unambiguous factors are selected and finally, the final model should also efficiently fit.

In addition, the interaction between the parameters can be clearly illustrated by response surface 3D plots (Dutta *et al.*, 2004) and also contour plots. RSM offers significant number of advantages compared to classical optimization method (Bas and Boyaci, 2007). First, RSM provides more information although with small number of experiments. Second, with RSM, the interaction between parameters can be study. In order to achieve optimization, RSM will reduce the number of trials (Gan *et al.*, 2007; Adinarayana and Ellaiah, 2002) and provide multiple regression approach (Gan *et al.*, 2007).

Besides, RSM also possess its negative part where the data needs to fit to second order polynomial but not all systems can be represented by second order polynomial (Bas and Boyaci, 2007). This problem can be eliminated by converting the data into other relevant form and can be explained by second order polynomial or if the second order

model make harder in explaining the system, narrowing the range of independent parameters.

Research by Bas and Boyaci (2007) also discovered that RSM cannot be used for optimization of all chemical and biochemical processes without any limitation and RSM also cannot be used in optimizing other purposes such as estimation of reaction kinetics. This is all because it is only usable for data that can be explained by second order model and not all systems can be fitted in second order model.

RSM is reported to be effective in optimizing various process parameters, levels of ingredients and formulation for variant products such as cassava cake (Gan *et al.,* 2007), adsorption of verofix red using biopolymer (Annadurai and Sheeja, 1998), purification of lipase (Gopinath *et al.*, 2003), extracellular protease production from *Pseudomonas* sp. (Dutta *et al.*, 2004) and extracellular alkaline protease production from *Aspergillus fischeri* (Saravanakumar *et al.*, 2010) have been reported by different researchers.

2.9 PROTEASE ASSAY

The extracellular production of microbial protease either from bacteria or fungal species had been successfully discovered by the researchers. The extracellular enzyme production is where the enzyme is secreted out of the cell and is abundant within the fermentation medium. In order to confirm the existence of this enzyme, the common method done by the researchers is protease enzyme assay.

Rong *et* al. (2010) said that every enzyme and its substrate can bind with each other at specific sites which possess complementary geometric shapes situated on the enzyme. So, assay is related to reaction between enzyme and its substrate by which the activity of the enzyme is being measured. Protease is already known to be a degradative enzyme that responsible in catalyzing the hydrolysis of protein (Rao *et al*., 1998). It can react with over board range of substrates either natural or synthetic substrates (Nirmal *et al*., 2011). Examples of natural substrates are keratin, gelatin, casein, albumin, collagen and elastin while azocasein is one of synthetic substrate (Nirmal *et al*., 2011).

Most of the researchers preferred in using casein as the substrate in the protease assay. Casein is a phosphoprotein that can be found in milk and this protein has been applied in variety of experimental application such as blocking agent in immunochemistry, recovery of enzyme activity from SDS extracted samples and also for the purposes of protease and kinase assays (Sigma-Aldrich Corp, 2006).

(Sigma-Aldrich Corp, 2011) had came out with a written protocol that described the standardized procedure of universal protease activity assay with the used of casein as substrate. Protease breaks down the peptide bond of larger proteins into smaller molecules. The protease digests the casein and amino acid Tyrosine together with several amino acids and peptide fragments are liberated from this reaction. After that, the existence of Tyrosine is quantified via spectrophotometric technique. This method was known as colorimetric and the principle of protease assay is explained by the following Equation 2.1 (Sigma-Aldrich Corp, 1999):

$$Casein + H_2O \xrightarrow{Protease} Amino\ Acids \quad (2.1)$$

Priya Chemicals (2010) had also proved the existence of several amino acids from the hydrolysis of casein. The data is clearly shown in Table 2.6.

Table 2.6: Amino acids composition in 100 g of Casein base

Protein Hydrolysate Solution (Casein base)	
	g/100 g Protein
L-Aspartic acid	13.00
L-Glutamic acid	25.39
L-Hydroxypoline	4.39
L-Valine	3.52
L-Isoleucine	3.68
L-Phenylalanine	3.47
L-Histidine	1.67
L-Threonine	4.77
L-Proline	1.47
L-Alanine	3.89
L-Methionine	1.72
L-Tryptophan	0.87
L-Arginine	2.09
L-Serine	4.85
L-Cystine	1.46
L-Leucine	5.98
L-Glycine	10.08
L-Tyrosine	3.31

Source: Prima Chemicals (2010)

The assay method proposed by Sigma-Aldrich Corp (2011) had been also applied by several researchers (Chow and Peticolas, 1948; Modler *et al.*, 1973; Morihara and Tsuzuki, 1977; Ogrydziak and Mortimer, 1977; Malik, 1985; Ray *et al*., 1992; Safarik and Safarikova, 1994; Samarntarn *et al*., 1999; Sandhya *et al*., 2005; Kumar *et al*., 2008; Paranthaman *et al*., 2009; Murthy and Naidu, 2010; Oyeleke, 2010; Benazir *et al*., 2011; Ranjbar *et al*., 2011; Sarkar *et al*., 2011). Some studies also used different substrates for protease assay such as Hemoglobin (Ray *et al*., 1992 and Ranjbar *et al*., 2011), Azocasein

(Mahmood *et al*., 1998; Charney and Tomarelli, 2011; Ranjbar *et al*., 2011), Azoalbumin (Andrade *et al*., 2002) and black gelatin (Safarik and Safarikova, 1994). Different type of substrates will be hydrolyzed to produce different type of product as standard.

Plate assay is also one of the protease assay method. Sandhya *et al*. (2005) did screening of neutral protease producers using plate medium. The growth medium contains casein as substrate for protease. The plate was then stained with Coomassie brilliant blue followed by destaining in order to observe the hydrolysis zone by measuring the radius of the hydrolysis zone due to neutral protease production.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The study is to implement shake flask fermentation for extracellular protease production using *Aspergillus niger* with the utilization of potato peel extract as substrate. Three parameters that might affect the extracellular protease production had also been studied.

3.2 CULTIVATION PROCESS

A fungal strain used in the present study was *Aspergillus niger* which kindly obtained from *Malaysian Agricultural Research and Development Institute* (MARDI), Selangor Darul Ehsan, Malaysia. The culture was grown on Potato Dextrose Agar (PDA) at 30℃ for a week for maximum growth and maintained at 4℃. The microorganism was periodically subculture by dispersing them using sterile inoculating loop onto fresh agar medium.

3.3 INOCULUM PREPARATION

The *Aspergillus niger* spores from the PDA slant were used for inoculums preparation before being used in inoculating the fermentation medium. 100 ml of inoculums broth prepared in the Erlenmeyer flask was inoculated with 2% of 1-week-old *Aspergillus niger* strain. Then, it was incubated at 30℃, 250 rpm for 24 hours.

3.4 SUBSTRATE PREPARATION

The substrates used in present study are potato peel extract. The potatoes were first washed to remove excess soil and manually peeled using the peeler to get the waste which was the peelings of the potatoes. The waste substrate was prepared by blending them in a blender with peel to water ratio of 1:3. The process proceeded with filtration. The substrates were frozen at -20℃ to prevent fluctuation due to seasonal variations of agricultural products. Once required, an amount of the substrates was thawed and used in the experiments.

3.5 SUBMERGED FERMENTATION

The fermentation media was first inoculated with 10% of inoculums broth. The fermentation was taken place in 250 ml Erlenmeyer flasks containing 100 ml of inoculated fermentation medium. The inoculated flasks were then incubated at 30℃ in the incubator shaker. The initial growth medium was composed of 2.4 g/l of KH_2PO_4, 2.0 g/l of NH_2Cl, 1.0 g/l of yeast extract, 1.0 g/l of potato peel extract, 0.5 g/l $MgSO_4$, 0.004 g/l $FeSO_4$ and glucose. The pH of the fermentation media was adjusted before sterilization via autoclaving.

3.5.1 Effect of pH on Protease Production

The effect of pH on protease activity produced by *Aspergillus niger* was carried out at different ranges of pH which were 3.5, 4.5, 5.5, 6.5 and 7.5. The pH of the medium was adjusted using 1M hydrochloric acid (HCl) or 1M sodium hydroxide (NaOH) in order to reach the particular pH respectively. Then, the prepared media were inoculated with inoculum prior to incubation for 72 hours at 30°C. After that, the assay was done and the protease activity was read at 660 nm.

3.5.2 Effect of Substrate Concentration on Protease Production

The effect of substrate concentration on protease activity produced by *Aspergillus niger* was done at different ranges of level of concentrations. The media were prepared by supplementing glucose of 20, 30, 40, 50 and 60 g/l. Then, the prepared media were inoculated with inoculum prior to incubation for 72 hours at 30°C. After that, the assay was done and the protease activity was read at 660 nm.

3.5.3 Effect of Agitation Speed on Protease Production

The effect of agitation speed on protease activity produced by *Aspergillus niger* was carried out at different ranges of agitation speed. Five sets of flask of the fermentation medium were incubated at 100, 150, 200, 250 and 300 rpm respectively. Then, the prepared media were inoculated with inoculum prior to incubation for 72 hours at 30°C. After that, the assay was done and the protease activity was read at 660 nm.

3.6 EXPERIMENTAL DESIGN AND PROCESS OPTIMIZATION

The statistical analysis for the production of extracellular protease enzyme by *Aspergillus niger* using potato peels extract as substrate was performed by using Design Expert V 6.0.8 software. Central Composite Design (CCD) was used to study the interaction of process variables by applying RSM (Bezerra *et al.*, 2008). In order to obtain the optimal protease activity, certain fermentation conditions such as pH (pH 3.5, 4.5, 5.5, 6.5 and 7.5), substrate concentrations (20, 30, 40, 50 and 60 g/l) and agitation speed (100, 150, 200, 250 and 300 rpm) were optimized. Each of these variables is varied over five levels which were low axial point (-α), low factorial point (-1), central point (0), high axial point (+α) and high factorial point (+1). Table 3.1 shows the list of range and coded level of fermentation process variables. The total number of experiments was 20. Table 3.1 shows the experimental range and level coded of process variables while Table 3.2 shows the design matrix of CCD for extracellular protease production. Protease activity (Y) was taken as the response of the design experiment. The full quadratic equation of the response variables for protease production was derived by using RSM as Equation 3.1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X \quad (3.1)$$

Where: $Y =$ responses; $\beta_0 =$ constant; $\beta_1, \beta_2, \beta_3 =$ linear regression; $\beta_{11}, \beta_{22}, \beta_{33} =$ interaction regression; $X_1, X_2, X_3 =$ variables.

Table 3.1: Experimental range and level coded of process variables

Independent variables	**Units**	**Symbol**	**Ranges and levels**				
			-α	**-1**	**0**	**+1**	**+α**
pH	-	X_1	2.14	3.50	5.50	7.50	8.86
Substrate concentration	g/l	X_2	6.36	20.00	40.00	60.00	73.64
Agitation speed	rpm	X_3	31.82	100.00	200.00	300.00	368.18

Table 3.2: Experimental design of CCD for extracellular protease production

Run	X_1 pH	X_2 Substrate Concentration (g/l)	X_3 Agitation Speed (rpm)
1	3.50	60.00	100.00
2	8.86	40.00	200.00
3	5.50	40.00	368.18
4	3.50	20.00	100.00
5	5.50	6.36	200.00
6	7.50	60.00	300.00
7	7.50	60.00	100.00
8	3.50	60.00	300.00
9	5.50	40.00	200.00
10	7.50	20.00	100.00
11	5.50	40.00	200.00
12	5.50	73.64	200.00
13	7.50	20.00	300.00
14	2.14	40.00	200.00
15	5.50	40.00	200.00
16	5.50	40.00	31.82
17	5.50	40.00	200.00
18	3.50	20.00	300.00
19	5.50	40.00	200.00
20	5.50	40.00	200.00

3.7 PREPARATION OF TYROSINE STANDARD CURVE

Tyrosine was prepared and used as the standard solution for protease assay. Five test tubes, labelled as Standard Blank, Standard 1, Standard 2, Standard 3 and Standard 4, were filed with 0, 5.0, 10.0, 25.0 and 50.0 µl of 1.1 mM Tyrosine accordingly and then were mixed with distilled water to the total amount of 250 µl of mixture in each test tube. After that, in every test tube, 625 µl of sodium carbonate and 125 µl of Folin & Ciocalteu's phenol reagent were added. Then, the contents in each test tube were mixed well and incubated at 37°C for 30 minutes. The incubated mixture was then checked using spectrophotometer for absorbance at 660 nm. The absorbance values were plotted against µg of tyrosine since 1 µmole of tyrosine is equivalent to 181 µg of tyrosine.

3.8 DETERMINATION OF TYROSINE (STANDARD CURVE)

The casein in buffer was incubated with the crude enzyme for 10 minutes at 37^{o}C. In order to stop the reaction, trichloroacetic acid was added. This will result in the production of trichloroacetic acid soluble peptides which contain tyrosine and trypthopan residues (Sigma-Aldrich Corp, 2005). Then, the mixture was centrifuged at appropriate speed and the supernatant was then mixed with sodium carbonate and Folin's reagent before further incubated at 30^{o}C for 30 minutes. For best results, the Folin's reagent should be added immediately after the addition of sodium carbonate. Sodium carbonate regulates any pH drop created by the addition of Folin's reagent. Tyrosine that reacts with Folin's reagent will produce color change (Sigma-Aldrich Corp, 2005). Folin's reagent will also react with other amino acids but to a lesser extent. The Tungsten and/or molybdate in the Folin's reagent will be reduced by the amino acid and generating one or more compounds with blue colored chromophore that can be determined colorimetrically at 660 nm (Sigma-Aldrich Corp, 2005). When more Tyrosine is released from casein, the blue coloured chromophore will be generated more meaning that the higher protease activity is achieved. The protease activity was measured spectrometrically in absorbance values and these obtained values will be compared to a standard curve of Tyrosine. The standard curve was generated by reacting known amount of Tyrosine with Folin's reagent to correlate the

changes in absorbance with the amount of Tyrosine. Referring to the standard curve, protease activity can be determined in terms of Units (amount of Tyrosine equivalents liberated from casein per minute).

3.9 PROTEASE ENZYME ASSAY

Protease activity was determined according to the method developed by Murthy and Naidu (2010) with slight modification. 200 µl of crude enzyme extract was added with 500 µl of 1% casein and 300 µl of 0.2 M phosphate buffer (pH 7.0). The reaction mixture was incubated at 37℃ for 10 minutes and then, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). After that, the mixture was centrifuged at 10,000×g for 15 minutes. The supernatant was then added with 5 ml of 0.4 M Sodium carbonate (Na_2CO_3) and 1 ml of diluted Folin & Ciocalteu's phenol reagent. Then, the resulting solution was incubated at room temperature for 30 minutes and the absorbance of the blue colour developed was read at 660 nm using spectrophotometer with Tyrosine standard. A unit of enzyme activity was defined as the amount of enzyme that released 1 µmole (181 µg) of Tyrosine per ml per minute under assay conditions. Protease activity was represented by the following Equation 3.2 in terms of Units/ml:

$$Units/ml = \frac{\mu mole\ of\ tyrosine\ (\mu mole) \times Total\ volume\ of\ assay(ml)}{Volume\ of\ sample\ (ml) \times Reaction\ time(min) \times Volume\ assayed(ml)} \quad (3.2)$$

3.10 CELL DRY WEIGHT

The cell biomass was determined according to the method developed by Ahmed *et al.* (2010), with slight modification. The resulting precipitate obtained after the centrifugation was filtered and washed trice to remove impurities and finally dried at 80℃ until constant weighed. The result was expressed by the following Equation 3.3 in g/l.

$$Cell\ Dry\ Weight = \frac{(Weight\ of\ dry\ filter + cell) - (Weight\ of\ dry\ filter)(g)}{Sample\ volume(l)} \quad (3.3)$$

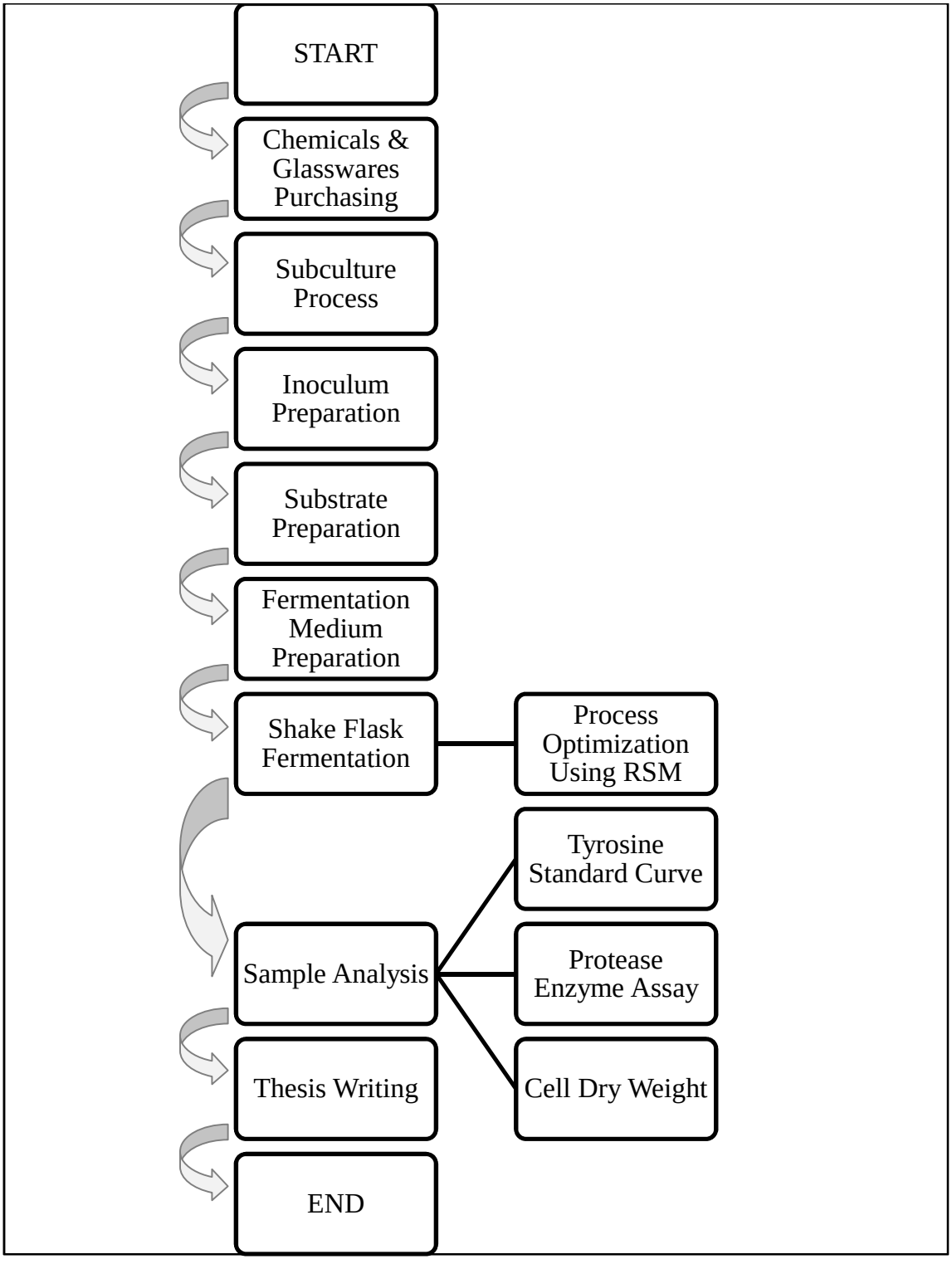


Figure 3.1: Overview of Research Process Flow

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 INTRODUCTION

All the related findings and their elaboration were included in this part. The experimental data were tabulated and presented in suitable tables/graphs and were being analysed in appropriate means. The obtained data was explained and if necessary, any supported evidences from the literature were also provided.

4.2 ONE FACTOR AT A TIME (OFAT) ANALYSIS

The fermentation process took place in the shake flask and the extracellular production of protease enzyme was being studied but certain parameters had been chosen for the purpose of optimization towards the production of protease enzyme. The best fermentation condition will probably produce maximum yield and that is why the appropriate selection of parameters' range is really important. Before proceed to optimization, all the chosen parameters to be optimized should undergo One Factor at a Time (OFAT) analysis. It is actually the conventional ways in doing optimization but in order to get the best range of parameters to be further use in the optimization process, it is vital to do so. The selected parameters were the pH of the fermentation medium, the concentration of substrate used and the agitation speed. In this study, the effect of those parameters towards the protease activity was examined. The afterwards subtopic will be discussed deeper by the obtained results from OFAT analysis.

4.2.1 Effect of pH

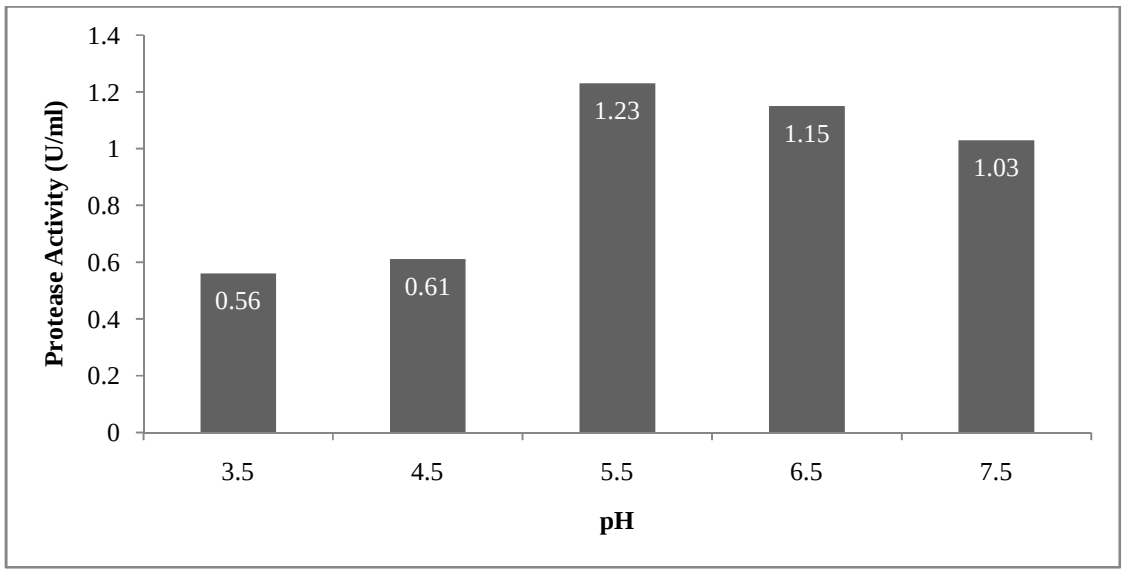


Figure 4.1: Effect of pH on Protease Production

Figure 4.1 illustrates the effect of pH on the production of protease enzyme in the submerged fermentation. It shows that the production of protease was strongly influenced by the pH changes although with just slight difference since it enhanced transport of various components across the cell membrane which then will encourage the growth of the cell and production of product (Sandhya *et al.*, 2005; Paranthaman *et al.*, 2009; Murthy and Naidu, 2010). Benazir *et al.* (2011) said that *Aspergillus niger* can produce protease over a pH range of 4.4 to 9 while this study found out that the protease enzyme showed its optimum activity, 1.23 U/ml, at pH 5.5. Decreasing the pH below 5.5 resulted in drastic decrease of protease activity while increasing the pH above 5.5 will also lower the protease activity but not too extreme. Referring to the result obtained, *Aspergillus niger* was said to be best producing acidic protease. Oyeleke *et al.* (2010) also reported other *Aspergillus sp.* that produced acidic protease which was *Aspergillus fumigatus* and managed to get 0.70 U/ml of protease activity at pH 5 but in this study, 1.23 U/ml was obtained at pH 5.5. This is might be due to the utilization of potato peel extract that enhanced the production of protease enzyme.

4.2.2 Effect of Substrate Concentration

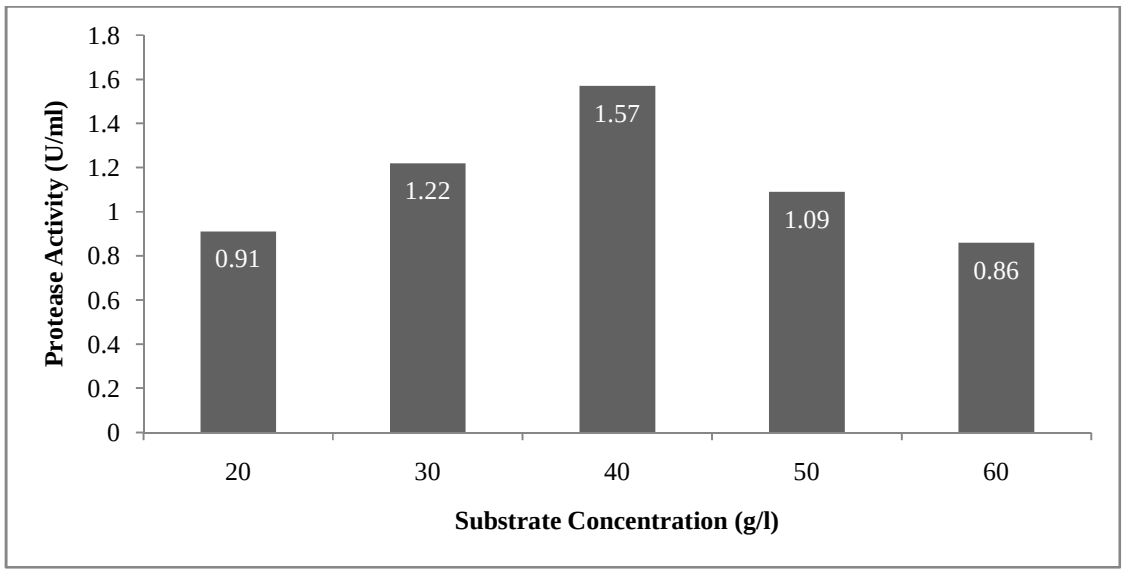


Figure 4.2: Effect of Substrate Concentration on Protease Production

Figure 4.2 shows the effect of substrate concentration on the production of protease enzyme but from the graph, it clearly showed that protease activity (1.57 U/ml) was at its optimum when 40 g/l of substrate was used. Andrade *et al.* (2002) also found out that 40 g/l of substrate concentration did show the maximum protease activity. Adequate supply of nutrient will enhance the cell growth and product production to the maximum. Both too low and too high concentration of carbon source will reduce protease activity. Low concentration of substrate provided insufficient nutrient to the cell and might be causing the cells to die and unable to further producing the protease enzyme while too high concentration of nutrient will repress the protease production and this statement was supported by Dekleva *et al.* (1985).

4.2.3 Effect of Agitation Speed

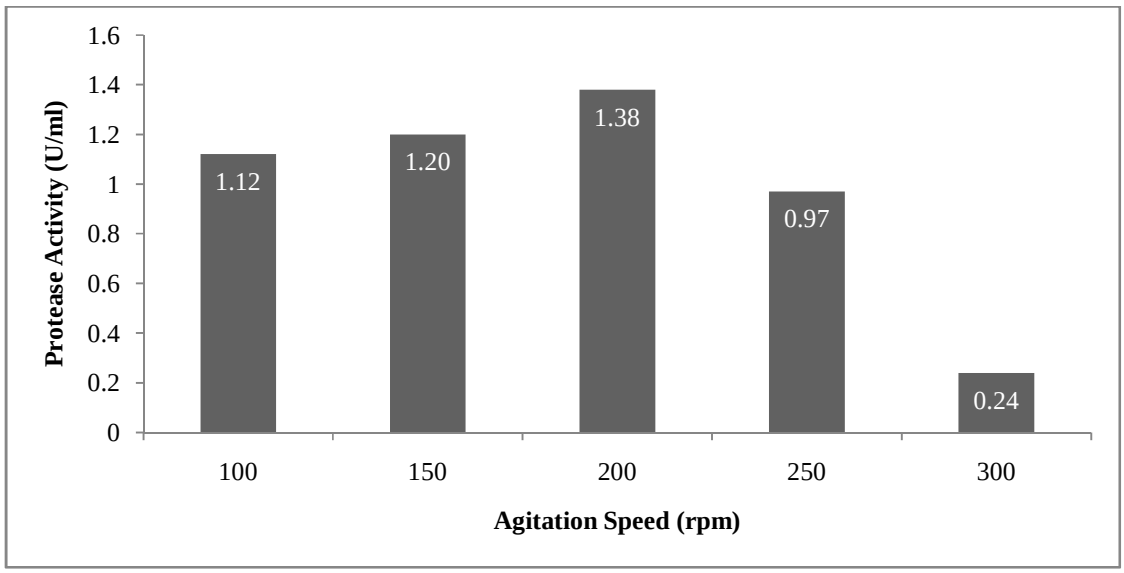


Figure 4.3: Effect of Agitation Speed on Protease Production

Figure 4.3 shows the effect of agitation speed towards the protease activity. From Figure 4.3 above, 200 rpm of rotational speed gave the optimum protease activity while at 100 rpm the protease activity was lower than the protease activity at 200 rpm. On the contrary, 300 rpm shows the lowest protease activity compared to the others. Results showed that agitation speed did affect the production of protease enzyme since it really helps in better mixing of the nutrients (Kamath *et al.*, 2010). Other researcher found out that agitation speed at 110 rpm and 130 rpm gave lower protease activity because the cells were unable to grow efficiently due to lack of aeration and nutrients uptake, meaning that the perfect mixing will liven up the cells to the maximum which also leads to produce optimum protease enzyme whereas higher agitation speed will results in microorganism and its morphological damages due to shear stress (Sepahy and Jabalameli, 2011). According to Singh *et al.*, (2011), agitation speed will also maintain the perfect mixing of oxygen throughout the fermentation medium for cell growth.

4.3 OPTIMIZATION USING RSM

The best range of parameters obtained from OFAT analysis will be bring forward for further optimization process. The optimization process will be facilitated by the usage of RSM. RSM provided the experimental design that will cover all points within the parameters range but with reduced number of runs.

4.3.1 RSM Model Fitting

The experimental results in Table 4.1 shows the effect of pH (X_1), substrate concentration (X_2) and agitation speed (X_3) on the production of extracellular protease enzyme. The protease activity (Y) was the response for this study. The Analysis of Variance (ANOVA) was performed in order to fit both manipulated variables and response to a quadratic model. ANOVA generated a regression equation in terms of protease activity (Y) as a function of pH, substrate concentration and agitation speed:

$$Y = 2.28 + 0.083X_1 + 0.13X_2 - 0.13X_3 - 0.60{X_1}^2 - 0.35{X_2}^2 - 0.71{X_3}^2 + 0.006587X_1X_2 - 0.012X_1X_3 + 0.025X_2X_3 \quad (4.1)$$

Table 4.1 also shows the predicted values based on equation 4.1 above while in Figure 4.4, the actual versus predicted values were plotted. The regression model was statistically analysed for ANOVA and gave out the results tabulated in Table 4.2. Through the ANOVA, the experimental results were analyzed to determine the lack of fit and the significant of the quadratic model and the effect of interaction between two manipulated variables. According to Gan *et al.* (2007), the lack of fit test is a failure measurement of a model to represent the experimental data at the point excluded in the regression while *de*Lima *et al.* (2010) discovered that the goodness-of-fit of the model was identified by the coefficient of determination (R^2) and it should at approximately 80%. In addition, it also said that P-value is a tool to check the significant of each coefficient. Greater F-value and smaller P-value represent better significant of the corresponding coefficient.

Table 4.1: RSM Experimental Design with the Actual and Predicted Protease Activities

Run	**X_1** **pH**	**X_2** **Substrate Concentration (g/l)**	**X_3** **Agitation Speed (rpm)**	**Y** **Actual Protease Activity (U/ml)**	**Predicted Protease Activity (U/ml)**
1	3.50	60.00	100.00	0.8411	0.7500
2	8.86	40.00	200.00	0.8231	0.7200
3	5.50	40.00	368.18	0.2236	0.0570
4	3.50	20.00	100.00	0.7308	0.5600
5	5.50	6.36	200.00	0.9021	1.0700
6	7.50	60.00	300.00	0.5608	0.7100
7	7.50	60.00	100.00	0.8944	0.9500
8	3.50	60.00	300.00	0.4511	0.5600
9	5.50	40.00	200.00	2.0442	2.2800
10	7.50	20.00	100.00	0.8624	0.7400
11	5.50	40.00	200.00	2.4329	2.2800
12	5.50	73.64	200.00	1.6329	1.4900
13	7.50	20.00	300.00	0.3247	0.4000
14	2.14	40.00	200.00	0.3112	0.4400
15	5.50	40.00	200.00	2.3051	2.2800
16	5.50	40.00	31.82	0.3045	0.5000
17	5.50	40.00	200.00	2.2785	2.2800
18	3.50	20.00	300.00	0.3460	0.2700
19	5.50	40.00	200.00	2.2359	2.2800
20	5.50	40.00	200.00	2.3849	2.2800

Table 4.2: ANOVA Analysis for Response Surface Quadratic Model

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob > F	
Model	12.68	9	1.41	41.840	< 0.0001	Significant
Residual	0.340	10	0.034			
Lack of Fit	0.240	5	0.049	2.640	0.1553	Not Significant
Pure Error	0.093	5	0.019			
Total	13.02	19				

R-Squared, R^2 (0.9741); Adjusted R-Squared (0.9508); Predicted R-Squared (0.8466); Adequate Precision (17.124)

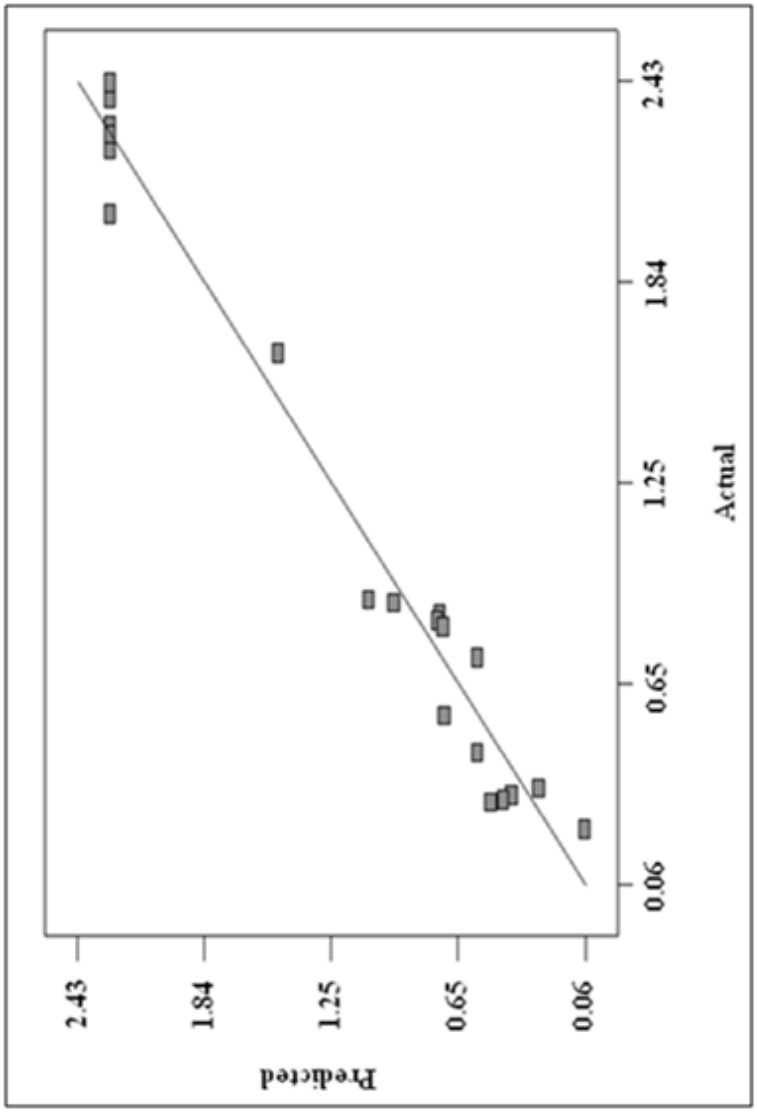


Figure 4.4: Plot of Predicted Value against Actual Value

According to Mimi Sakinah *et al.* (2009), the F-value is the fraction between mean square of the regression model and the mean square of the error. The F-value of the regression model is 41.84 which indicated that the quadratic model of the protease activity were statistically significant at 95% confidence level. It suggested that the data represented by the model is significantly higher than the non-interpreted data. This large F-value might occur due to noise for only a 0.01% chance. The value of P<0.05 should less than 0.50 indicates that the model terms is significant and the result shows the significance of the regression model for having Probability<F less than 0.0001.

The lack of fit for the response shown was insignificantly relative to the pure error. There is only 15.53% of chance that the lack of fit could be this large due to noise. The non-significant lack of fit is good as it is highly recommended for the model to be fit.

The R^2 value should always between 0 and 1 and the closer the R^2 value to 1, the better the correlation between actual and predicted values (Li *et al.*, 2006). R^2 exceeding 80% indicated a high proportion of variability was explained well by the model (Varnalis *et al.*, 2004) and the R^2 values for the response were around 97.41% indicating that 97.41% of the data can be explained by the model but only small portion of 2.59% of the data cannot be interpreted by the model so, this model indicated close agreement between both the actual and predicted values by the model. The predicted R^2 of 0.8466 was in reasonable agreement with the adjusted R^2 of 0.9508 since the difference is less than 0.2 (Mimi Sakinah *et al.*, 2009). Singh *et al.* (2011) said that higher adjusted R^2 indicates greater significance of the model.

The adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. With 17.124 of adequate precision, which was highly desirable and indicated an adequate signal, the model can be used to navigate the design space.

4.3.2 Interaction of Parameters

RSM came out with 3-Dimensional curve that really help in understanding the effects of interaction between several fermentation parameters and it also provided the optimum values for each parameter that required for maximum protease production.

Since there were three parameters being studied, three 3D response surface plots of interaction of parameters will be generated. Figure 4.5 represent the interaction between pH-substrate concentration while Figure 4.6 and Figure 4.7 represent the interaction between pH-agitation speed and substrate concentration-agitation speed, respectively. In each set of 3D response surface plot, two variables varied within the experimental range while the other one variable remained constant at zero level (Singh *et al.*, 2011).

4.3.2.1 Effect of pH and Substrate Concentration

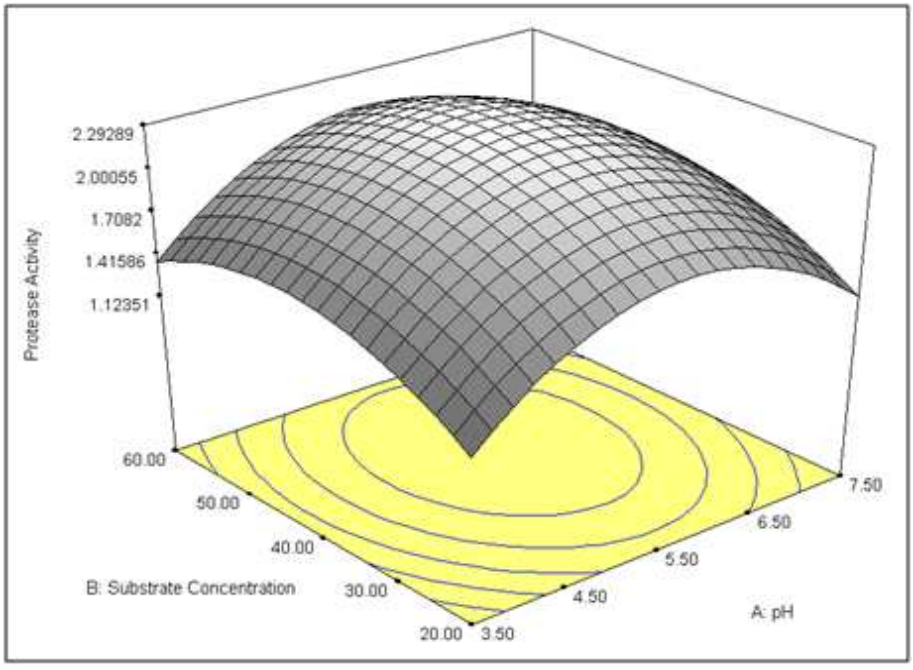


Figure 4.5: 3D Response Surface Plot of pH and Substrate Concentration Interaction

Figure 4.5 above illustrates the protease production with respect to pH against substrate concentration and it really showed that pH and substrate concentration strongly influenced the protease activity. Actually the hydrogen (H^+) concentration attributed the effect of microbial growth. H^+ can act as substrate at neutral pH while can be inhibitor at both acidic and alkaline condition. From the interaction, the protease activity increased with the increase of pH and substrate concentration up to pH 5.50 and 40 g/l of substrate, respectively. The decreasing of protease activity was seen when the pH greater than 5.50 while the substrate concentration greater than 40 g/l. Murthy and Naidu (2010) also reported that the increase of pH of the substrate will decrease the enzyme activity and different species of microorganisms will vary in the activity of enzyme depending on metabolism. Naturally any changes in pH may affect the protein structure and a reduce enzyme activity exceeded the optimum ph was due to inactivation of the enzyme or the enzyme is instability (Natarajan and Rajendran, 2009). Benazir *et al.* (2011) said that *Aspergillus niger* can produce protease over a pH range of 4.4 to 9 which somehow supported the data for this study.

The maximum protease production was at pH 5.50 and substrate concentration of 40 g/l. The microorganism is allowed to reach the exponential growth phase once the substrate is being utilized. Product formation is somehow repressed by either metabolites or by the main product itself. It shows that normally the product formation decrease when the substrate concentration increase (Escobar and Barnett, 1993). In this study, the protease production was high at 40 g/l which was between 20 and 60 g/l. Too low substrate concentration will results in inadequate nutrients supply to the cells and tended to affect its growth and product formation. Lower concentration of carbon source is also reported not suitable for enzyme production by *Aspergillus japonicas* (Beverini *et al.*, 1990). Too high substrate concentration also showed great affect in prohibiting enzyme activity. Other research reported the similar finding with this study where the enzyme production was optimum at 40 g/l (Andrade *et al.*, 2002) and indirectly supported the results of this study.

4.3.2.2 Effect of pH and Agitation Speed

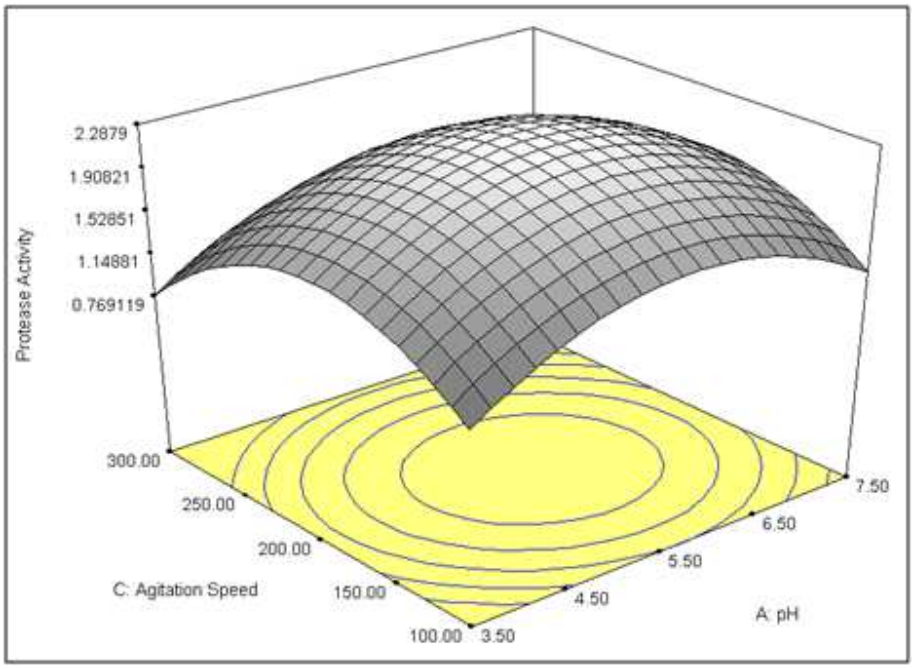


Figure 4.6: 3D Response Surface Plot of pH and Agitation Speed Interaction

Referring to Figure 4.6, the increase of pH up to 5.5 depicted the increase of protease activity while the increase of agitation speed from 100 rpm up to 200 rpm also resulted in increasing protease activity. Freire *et al.* (1997) and Alonso *et al.* (2005) also reported the optimum agitation speed of 200 rpm which support the finding of this study. When the pH and the agitation speed started to increase above pH 5.50 and 200 rpm, respectively, the production of protease showed its reduction. The increase of agitation speeds will frequently damaged the cells and resulted in changing the morphology of the cells (Sepahy and Jabalameli, 2011; Singh *et al.*, 2011). This was due to shear stress from the effect of agitation speed and leaded to slow down the protease activity. However, if the speed was relatively low, the oxygen will not enter the medium and not equally distributed throughout the medium. Then, the cells died in the anaerobic condition and protease production became retarded. This shows that shaking of aerobic culture is vital in the growth and protease production as it maintain proper oxygen transfer and the mixing of

growing cells (Singh *et al.*, 2011). The maximum protease activity was seen at pH 5.50 and 200 rpm. Many other researchers also reported a range of 150 – 300 rpm of optimum agitation speed for protease production from different isolates such as *Bacillus brevis* (Banerjee *et al.*, 1999), *Bacillus horikoshii* (Joo *et al.*, 2002) *Arthrobacter ramosus* and *Bacillus alkalophilus* (Kanekar *et al.*, 2002).

4.3.2.3 Effect of Substrate Concentration and Agitation Speed

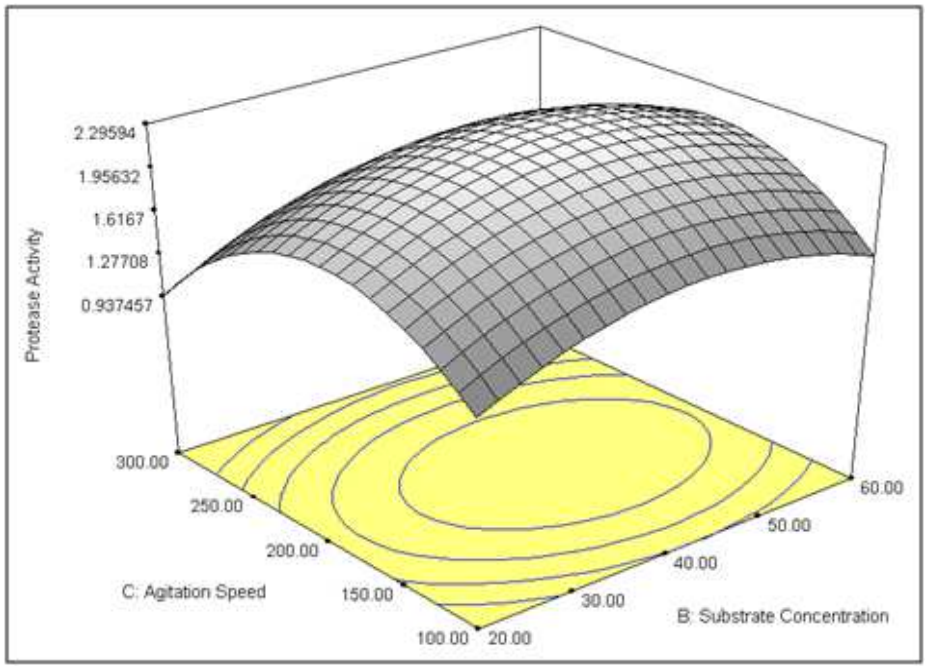


Figure 4.7: 3D Response Surface Plot of Substrate Concentration and Agitation Speed Interaction

The interaction between substrate concentration and agitation speed can be clearly seen in Figure 4.7. Still, the increase of substrate concentration and agitation speed up to 40 g/l and 200 rpm, respectively, showed the increase of protease production while once both of the substrate concentration and agitation speed level higher than 40 g/l and 200 rpm, respectively, the protease activity will start to decrease again. The result showed that the optimum protease production can be achieved at 40 g/l of substrate concentration and 200

rpm of agitation speed. Perfect mixing of oxygen and adequate supply of substrate will end up with optimum protease production.

4.3.3 Validation of the Model

The prediction made by the model said that the maximum protease production can be obtained by using the RSM optimized condition of parameters. Verification of the model is to validate the adequacy of the equation model and this is achieved by once again run the experiment under optimized condition suggested by the RSM. There were three verifications experiments to be carried out and the details were tabulated in the following Table 4.3. The shake flask fermentation was done at pH of 5.50, substrate concentration of 40 g/l and 200 rpm of agitation speed. These results corroborate the predicted values and the effectiveness of the model (Saravanakumar *et al.*, 2010).

The maximum protease activity was 2.2356 U/ml which closed to the predicted values by RSM. This verified the validity of the model and existence of the optimal point with error from 0.0250 to 0.0929.

Table 4.3: Construction Model and Data Validation for Protease Enzyme Activity

Run	**Optimized Parameter**			**Protease Activity (U/ml)**		**Error**
	pH	**Substrate Concentration (g/l)**	**Agitation Speed (rpm)**	**Actual**	**Predicted**	
1	5.50	40	200	2.1871	2.2800	0.0929
2	5.50	40	200	2.2648	2.2800	0.0152
3	5.50	40	200	2.2550	2.2800	0.0250

4.4 RELATIONSHIP BETWEEN CELL DRY WEIGHT AND PROTEASE ENZYME PRODUCTION

After the four days of fermentation, the weight of biomass was determined in order to observe the growth profile of *Aspergillus niger* in the fermentation medium by plotting the cell dry weight against the time of fermentation. Along the way, the production of protease enzyme was also observed and the similar graph was obtained by plotting the OD reading at the secondary axis against the time of fermentation. Both graphs are shown as Figure 4.8 below.

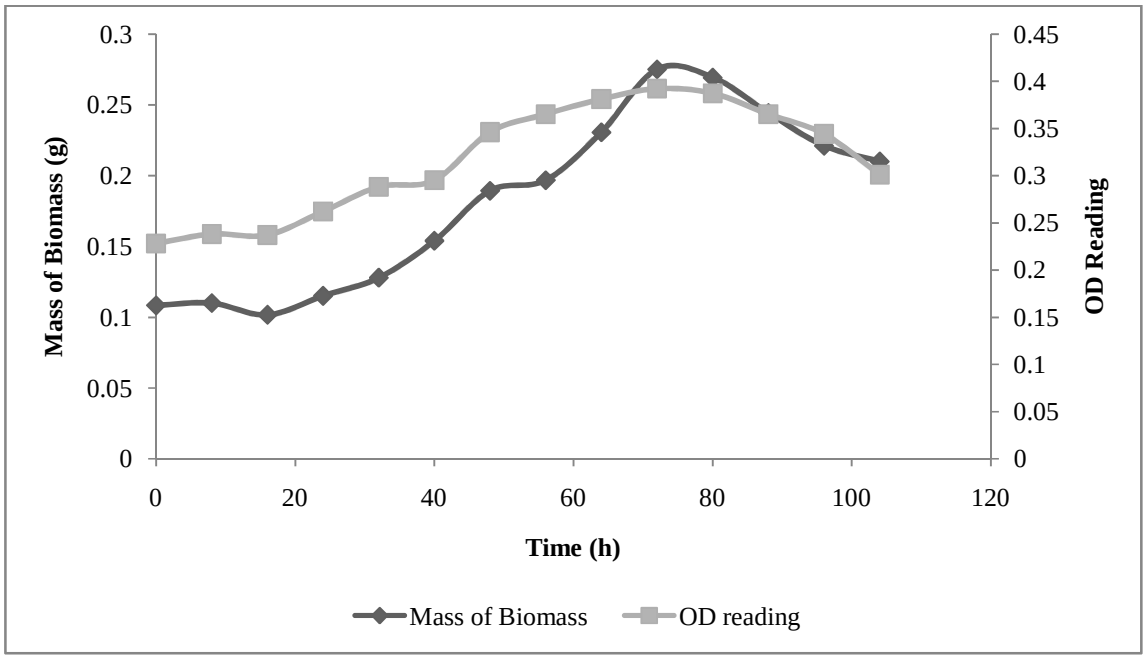


Figure 4.8: Growth profile of *Aspergillus niger* and protease enzyme production

For the cell dry weight analysis, the samples were taken every 8 hours starting from 0 hour to 104 hours. Then, the biomass was obtained by filtration before drying at 80 °C. After that, the cell dry weight was determined. The data was analysed and represented by the following Figure 4.8. It clearly shows the profile of biomass growth within the fermentation medium.

Within the first 16 hours, the growth of biomass seems to be none and this phase was normally known as the lag phase. At this phase, the fungi still did not grow but in the process of adaptation with the surrounding. Right after the 16 hours of fermentation, the mass of biomass started to significantly increased. It is called the phase of exponential and also known as log phase. Basically at this phase, the fungi used up the nutrient provided from the fermentation medium and started to grow, reaching its maximum growth. The biomass was found growing at its maximum at the 72 hours of fermentation. Next, the fungi faced stationary phase where weight of biomass was quite maintained and finally started to decrease from the 88 hours, indicated the death phase of the fungi.

On the same time, the supernatant from the fermentation was analysed for the extracellular protease production represented by OD reading. From Figure 4.8, the enzyme was generated along the exponential phase of *Aspergillus niger*. It proved that the protease enzyme is the primary metabolite as it was produced during the active growth of the fungi and this enzyme production was growth associated.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

In this part, the overall conclusion about the study was done and some recommendations are pointed out for the improvement of protease production study in the future.

5.2 CONCLUSION

As the conclusion, the study of the extracellular protease enzyme production has been successfully done in the submerged fermentation process by *Aspergillus niger* with the use of potato peel extract as the additional carbon source. One-Factor-at-a-Time (OFAT) analysis was done in order to select the significant parameters that affect protease production. From the analysis, three selected parameters did show great influence towards protease production. The three parameters and its range were pH of the fermentation medium (pH 3.5 – pH 7.0), the substrate concentration (40 – 60 g/l) and the agitation speed (100 -200 rpm). Protease enzyme showed the optimum activity at pH 5.50 with 1.23 U/ml while at 40 g/l the optimum protease activity was also obtained with 1.57 U/ml. An optimum protease activity was also shown at 200 rpm with 1.38 U/ml. With the use of Response Surface Methodology (RSM), the optimization process became much easier since it helped in doing the statistical analysis. It came out with a model that correlates the three parameters for high yield of protease activity. Furthermore, the better understanding on the effect of interaction between parameters was also achieved since it provided the 3D

response surface plots. The results from RSM depicted that pH 5.50, 40 g/l of substrate concentration and 200 rpm were the optimum parameters and yielded protease activity of 2.2356 U/ml. Besides, the utilization of potato peel extract was highly recommended due to its potent of becoming cost-effective carbon source.

5.3 RECOMMENDATIONS

First, the utilization of agriculture and food industrial wastes as the cost-effective substrates should be further being studied especially on the usage of potato peel extract but the exact chemical composition (carbohydrate, starch, protein, etc.) of the extract should be initially determined first. In the future, an optimization of potato peel extracts concentration as the main carbon source can also be done. Since nowadays the solid state fermentation was reported in producing high yield of various products such as pigments, biocellulose etc if compared to submerged fermentation so it might be relevant to be implemented in this study.

Second, application of other new technology can somehow improve the production of protease. Many researchers mostly focus on the optimization of fermentation parameters in the production of enzyme. Besides, the application of genetic engineering in the enzyme production should be promising for high yield enzyme production due to the alteration of microbial genetic that will force the microorganisms to produce more enzymes.

Third, this small scale of protease production study can be further scale up with the utilization of bioreactor. Compared to shake flask fermentation process, the agitation and aeration processes can be regulated in the bioreactor. The small production of protease activity in shake flask fermentation is maybe due to the limited aeration within the fermentation medium and causes the death of the cells. In addition, bioreactor makes the sterilization and sampling processes become easy since within the bioreactor, the sterilization can be done directly.

Forth, from the crude protease enzyme obtained from this study, it is recommended for further purification of the enzyme. The enzyme purification process is promising in high productivity but is relatively costly. This purified protease enzyme is likely more suitable to be used by human and in other industries that deal with human's life such as pharmaceutical and food industries rather than using the crude protease enzyme. Purified enzyme is safer since within the purification process other contaminants are eliminated leaving the clean enzyme. Moreover, toxicology study on the protease enzyme can also be done since the enzyme will be someday being consumed by the human being. This procedure is to make sure the efficacy and the safety of the enzyme.

REFERENCES

Adinarayana, K., and Ellaiah, P. (2002). Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus sp. Journal of Pharmacy and Pharmaceutical Sciences*. 5(3): 272-278.

Adinarayana, K., Ellaiah, P. and Prasad, D. S. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *Americal Association of Pharmaceutical Scientists Pharmaceutical Science and Technology.* 4(4): 440-448.

Ahmed, I., Irfan, M., Nadeem, M., Zia, M. A., Ahmad, B. M., and Iqbal, H. M. (2010). Optimization of media and environmental conditions for alkaline protease production using *bacillus subtilis* in submerged fermentation process. *International Journal of Agro Veterinary and Medical Sciences*. 4(4): 105-113.

Alonso, F. O. M., Oliveira, E. B. L., Dellamore-Oritz, G. M. and Pereira-Meirelles, F. V. (2005). Improvement of lipase production at different stirring speeds and oxygen levels. *Brazillian Journal of Chemical Engineering*. 22(1): 9-18.

Andrade, V. S., Sarubbo, L. A., Fukushima, K., Miyaji, M., Nishimura, K., and Campos-Takaki, G. M. (2002). Production of extracellular proteases by *mucor circinelloides* using d-glucose as carbon souces/substrate. *Brazillian Journal of Microbiology*. 33: 106-110.

Annadurai, G. and Sheeja, R. Y. (1998). Use of Box-Behnken design of experiments for the adsorption of verofix red using biopolymer. *Bioprocess Engineering*. 18: 463-466.

Arapoglou, D., Vlyssides, A., Varzakas, T. H., Haidemenaki, K., Malli, V., Marchant, R. and Israilides, C. (2009). Alternatives ways for potato industries waste utilisation. *Proceedings of the 11th International Conference on Environmental Science and Technology* (pp B54-B61). Chania, Crete, Greece: SRCosmos.

Banerjee, C. U., Sani, R. K., Azmi, W. and Soni, R. (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochemistry*. 35: 213-219.

Bas, D. and Boyaci I. H. (2007). Modelling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*. 78: 836-845.

Benazir, J. F., Suganthi, R., Anjana, H., Ramesh, K. V., Aswathi, M. P., Niraimathi, G., Mala, M. K., Sukanya, S. and Santhi, R. (2011). Bioindustrial of agroindustrial waste in solid state fermentation by *Aspergillus niger* for the production of protease. *Asiatic Journal of Biotechnology Resources*. 2(04): 422-435.

Beverini, M. and Metche, M. (1990). Identification, purification and physicochemical properties of tannase of *Aspergillus oryzae*. *Sciences des Aliments.* 10: 807-816.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., and Escaleira, L., A (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta.* 76: 965 – 977.

Calik, P., Calik, G.and Ozdamar, T. (1998). Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis*: use of citric acid as the carbon source. *Enzyme and Microbial Technology*. 23: 451-461.

Chakrabarthi, S. K., Matsumura, N. and Ranu, R. S. (2000). Purification and characterization of extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). *Current Microbiology*. 40: 239-244.

Charney, J. and Tomarelli, R. M. (1947). A colorimetric method for the determination of the proteolytic activity of deodenal juice. *The Journal of Biological Chemistry*. 171: 501-505.

Chow, B. F. and Peticolas, M. A. (1948). A rapid method for the determination of proteolytic activities of enzyme preparations. *A Journal of General Physiology*. 17-24.

Coral, G., Arikan, B., Unaldi, M. N. and Guvenmes, H. (2002). Purification and characterization of protease-resistant cellulase form *Aspergillus niger*. *Journal of Fermentation and Bioengineering*. 39: 122-127.

Dekleva, M. L., Titus, J. A. and Strohl, W. R. (1985). Nutrient effects on anthracycline production by *Streptomyces peuticus* in a defined medium. *Canadian Journal of Microbiology*. 31: 287-294.

*de*Lima, C. J. B., Coelho, L. F., and Contiero, J (2010). The use of response surface methodology in optimization of lactic acid production: focus on medium supplementation, temperature and pH control. *Food Technology and Biotechnology.* 48(2): 175 – 181.

Ducros, E., Ferrari, M., Pellegrino, M., Raspanti, C. and Bogni, C. (2009). Effect of aeration and agitation on the protease production by *Staphylococcus aureus* mutant RC128 in stirred tank bioreactor. *Bioprocess and Biosystem Engineering.* 32: 143-148.

Dunne, C., Crowley, J. J., Moenne-Loccoz, Y., Dowling, D. N., *de*Bruijn, F. J. and O'Gara, F. (1997). Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. *Microbiolog.,* 143: 3921-3931.

Dutta, J. R., Dutta, P. K. and Banerjee, R. (2004). Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. *Process Biotechnology*. 39: 2193-2198.

El Enshasy, H., Abuoul-Enein, A., Helmy, S., and El Azaly, Y. (2008). Optimization of the industrial production of alkaline protease by *Bacillus Licheniformis* in different production scales. *Australian Journal of Basic and Applied Sciences*. 2(3): 583-593.

Escobar, J. and Barnett, S. M. (1993). Effect of agitation speed on the synthesis of *Mucor miehei* acid protease. *Enzyme and Microbiasl Technology.* 15: 1009-1013.

Food and Drug Administration (FDA). (2001). GRAS Notification – Exemption claim for ten microbially-derived enzyme preparations that are the sucject of a GRAS affirmation petition, GRASP 3G0016 [Data file]. Available from http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn0089.pdf.

Freire, D., Geraldo, L. and Alves, T. (1999). Mathematical modeling of lipase and protease production by *Penicillium restrictum* in a batch fermenter. *Applied Biochemistry and Biotechnology*. (77-79): 845-855.

Freire, D. M. G., Teles, E. M. F., Bon, E. P. S. and SantAnna, G. L. (1997). Lipase production by *Penicillium restrictum* in a laboratory-scale fermenter: media composition, agitation and aeration. *Appl.ied Biochemistry and Biotechnology.* 63: 409-421.

Gan, H. E., Karim, R., Muhammad, S. K. S., Bakar, J. A., Hashim, D. M. and Rahman, R. A. (2007). Optimization of the basic formulation of a traditional baked cassava cake using response surface methodology. *Lebensmittel-Wissenschaft und-Technologie* , 40, 611-618

Global Industry Analysts. (2011). Global industrial enzymes market to reach US$3.74 billion by 2015, according to a new report by Global Industry Analysts, Inc. Retrieved on Febuary 3, 2011 from http://www.prweb.com/releases/ industrial_enzymes/proteases_ carbohydrases/prweb8121185.htm.

Gombert, A. K., Pinto, A. L., Castilho, L. R. and Freire, D. M. G. (1999). Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. *Process Biochemistry*. 35: 85-90.

Gopinath, S. C. B., Hilda, A., Priya, T. L., Annadurai, G. and Anbu, P. (2003). Purification of lipase from *Geotrichum candidum*: conditions optimized for enzyme production using Box-Behnken design. *World Journal of Microbiology & Biotechnology*. 19: 681-689.

Gregori, A., Svagelj, M., and Pohleven, J. (2007). Cultivation techniques and medicinal properties of *Pleurotus sp*. *Food Technology and Biotechnology*. 45(3): 236-247.

Hwang, Y. B., Lee, A. C., Chang, H. N. and Chang, Y. K. (1991). Dissolved oxygen concentration regulations using auto tuning proportional integral derivative controller in fermentation process. *Biotechnology Techniques*. 5: 85-90.

Ikram-Ul-Haq, Mukhtar, H., and Umber, H. (2006). Production of protease by *Penicillium chrysogenum* through optimization of environmental conditions. *Journal of Agriculture and Social Sciences*. 2(1): 23-25.

Joo, H. S., Kumar, C. G., Park, G. C., Kim, K. T., Paik, S. R. and Chang, C. S. (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. *Process Biochemistry*. 38: 155-159.

Kamath, P., Subrahmanyam, V. M., Rao, J. V. and Raj, P. V. (2010). Optimization of cultural conditions for protease production by a fungal species. *Indian Journal of Pharmaceutical Sciences*. 72(2): 161-166.

Kanekar, P. P., Nilegaonkar, S. S., Sarnaik, S. S.and Kelkar, A. S. (2002). Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresource Technology*. 85: 87-93.

Khuri, A. I. (Ed.). (2006). Response Surface Methodology and related topics. Singapore: World Scientific Publishing Co. Pte. Ltd.

Kirk, O., Borchert, T. V. and Fuglsang, C. C. (2002). Industrial enzyme applications. *Current Opinion in Biotechnology*. 13: 345-351.

Kumar, P. K. P., Mathivanan, V., Karunakaran, M., Renganathan, S. and Sreenivasan, R. S. (2008). Studies on the effects of pH and incubation period on protease production by *Bacillus* sp. using groundnut cake and wheat bran. *Indian Journal of Science and Technology*. 1(4): 1-4.

Li, Y., Lin, J., Meng, D., Lu, J., Gu, G. and Mao, Z. (2006). Effect of pH, cultivation time and substrate concentration on the endoxylanase production by *Aspergillus awamori* ZH-26 under submerged fermentation using central composite rotary design. *Food Technology and Biotechnology*. 44(4): 473-477.

Mahmood, A. U., Greenman, J., and Scragg, A. H. (1998). Orange and potato peel extracts: analysis and use as *Bacillus* substrates for the production of extracellular enzymes in continuous culture. *Enzyme and Microbial Technology*. 22: 130-137.

Malik, R. K., Prasad, R. and Mathur, D. K. (1985). Effect of some nutritional and environmental factors on extracellular protease production by *Pseudomonas* SP.B-25. *Le Lait*. 65: 169-183.

Mimi Sakinah, A. M., Ismail, A. F., Hassan, O., Zularisam, A. W. and Rosli, M. D. (2009). Influence of starch pretreatment on yield of cyclodextrins and performance of ultrafiltration membranes. *Desalination*. 239: 317-333.

Modler, H. W., Brunner, J. R. and Stine, C. M. (1973). Extracellular protease of *Penicillium roqueforti*. *Journal of Diary Science*. 57(5): 523-527.

Morihara, K. and Tsuzuki, H. (1977). Production of protease and elastase by *Pseudomonas aeruginosa* strains isolated from patients. *Infection and Immunity*. 15(3): 679-685.

Murthy, P. S., and Naidu, M. M. (2010). Protease production by *Aspergillus oryzae* in solid-state fermentation utilizing coffee by-products. *World Applied Sciences Journal*. 8(2): 199-205.

Natarajan, K. and Rajendran, A. (2009). Effect of fermentation parameters on extracellular tannase production by *Lactobacillus plantarum* MTCC 1407. *E-Journal of Chemistry*. 6(4): 979-984.

Nigam, P. S., and Pandey, A. (2009). Solid-state fermentation technology for bioconversion of biomass and agricultural residues. In *Biotechnology for Agro-Industrial Residues Utilisation: Utilisation of Agro-Residues.* Springer.

Nirmal, N. P., Shankar, S. and Laxman, R. S. (2011). Fungal proteases: an overview. *International Journal of Biotechnology and Biosciences.* 1(1): 1-40.

Ogrydziak, D. M. and Mortimer, R. K. (1977). Genetics of extracellular protease production in *Saccharomycopsis lipolytica*. *Genetics*. 87: 621-632.

Oyeleke, S. B., Egwim, E. C., and Auta, S. H. (2010). Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production. *Journal of Microbiology and Antimicrobials*. 2(7): 83-87.

Paranthaman, R., Alagusundaram, K., and Indhumathi, J. (2009). Production of protease from rice mill wastes by *Aspergillus niger* in solid-state fermentation. *World Journal of Agricultural Sciences*. 5(3): 308-312.

Priya Chemical. (2010). Casein Base and Soya Base. Retrieved on September 7, 2011 from http://www.priyachem.com/casesoy.htm.

Purwanto, L. A., Ibrahim, D., and Sudrajat, H. (2009). Effect of agitation speed on morphological changes in *Aspergillus niger* hypae during production of tannase. *World Journal of Chemistry*. 4(1): 34-38.

Ranjbar, M., Sendi, J. J. and Zibaee, A. (2011). Proteolytic activity in the midgut of *Ectomyelois ceratoniae* Zeeler (Lepidoptera: Pyralidae), pomegranate carob moth. *Invertebrate Survival Journal*. 8: 132-142.

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*. 62(3): 597-635.

Ray, M. K., Devi, K. U., Kumar, G. S. and Shivaji, S. (1992). Extracellular protease from the antarctic yeast *Candida humicola*. *Applied and Environmental Microbiology*. 58(6): 1918-1923.

Hoffmann, R. (2010). Microbe fact sheet: *Aspergillus niger*. Microbe Inotech Blog. Retrieved on February 20, 2011 from http://blog.microbeinotech.com/bid/49873/ Microbe-Fact-Sheet-Aspergillus-niger.

Rong, P., Zhang, X. J., Zhang, Z. J., Yuan, Z., Tian, W. X. and He, R. Q. (2010). Substrate-induced changes in protease active site conformation impact on subsequent reactions with substrates. *The Journal of Biological Chemistry*. 285(30): 22950-22956.

Safarik, I. and Safarikova, M. (1994). Spectrophotometric determination of effective proteolytic activity in biodetergents. *Journal of Biochemical and Biophysical Methods.* 28: 131-136.

Salahuddin, P., and Khan, A. U. (2008). Proteolytic enzymes database. *Journal of Proteomics and Bioinformatics*. 1(2): 109-111.

Samarntarn, W., Cheevadhanarak, S. and Tanticharoen, M. (1999). Production of alkaline protease by a genetically engineered *Aspergillus oryzae* U1521. *Journal of General and Applied Microbiology.* 45: 99-103.

Sambamurthy, K., and Kar, A. (2006). Proteases [Proteolytid Enzymes]. In *Pharmaceutical Biotechnology* (pp. 344-351). New Delhi: New Age International (P) Ltd., Publishers.

Sandhya, C., Sumantha, A., Szakacs, G. and Pandey, A. (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid state fermentation. *Process Biotechnology*. 40: 2689-2694.

Saravanakumar, K., Thiyagarajan, A., and Kaviyarasan, V. (2010). Optimization of medium constituents for the production of extracellular alkaline Protease by *Aspergillus fischeri* using response surface experimental design. *Journal of Biosciences Research*. 1(3): 118-129.

Sarkar, P., Mukesh. M. and Singh, R. (2011). Microbial consortium: a new approch in effective degradation of organic kitchen wastes. *International Journal of Environmental Science and Development*. 2(3): 1-5.

Sepahy, A. A. and Jabalameli, L. (2011). Effect of culture conditions on the production of an extracellular protease by *Bacillus sp.* isolated from soil sample of Lavizan Jungle Park. *Enzyme Research*. 2011(219628): 1-7.

Sidkey, N. M., Abo-Shadi, M. A. A., Al-Mutrafy, A. M., Sefergy, F. and Al-Reheily, N. (2010). Screening of microorganisms isolated from some enviro-agro-industrial wastes in Saudi Arabia for amylase production. *Journal of American Science*. 6(10): 926-939.

Sigma-Aldrich Corp. (1999). Enzymatic assay of protease. Retrieved on October 18, 2011 from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/c7926enz.Par.0001.File.tmp/c7926enz.pdf.

Sigma-Aldrich Corp. (2005). Protease colorimetric detection kit. Retrieved on October 18, 2011 from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/pc0100bul.Par.0001.File.tmp/pc0100bul.pdf.

Sigma-Aldrich Corp. (2006). Casein sodium salt from bovine milk. Retrieved on October 19, 2011 from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/c8654pis.Par.0001.File.tmp/c8654pis.pdf.

Sigma-Aldrich Corp. (2011). Universal protease activity assay: casein as a substrate. Retrieved on October 18, 2011 from http://www.sigmaaldrich.com/life-science/learning-center/life-science-video/universal-protease.html.

Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K. and Garg, S. K. (2011). Comparative One-Factor-at-a-Time, Response Surface (Statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a phychrotrophic *Pseudomonas putida* SKG-1 isolate. *Microbial Cell Factories*. 10(1): 114.

Smythe, C. V. (1950). Microbiological production of enzymes and their industrial applications. *Economic Botany*. 126-144.

Sumantha, A., Larroche, C., and Pandey, A. (2006). Microbiology and Industrial biotechnology of food-grade proteases: a perspective. *Food Technology Biotechnology*. 44(2): 211-220.

Thomas, C. R. (1990). Problems of shear in biotechnology. In: Winkler, M. A. (Ed.). *Chemical Engineering Problems in Biotechnology* (pp 23-92). London: Elsevier Applied Science.

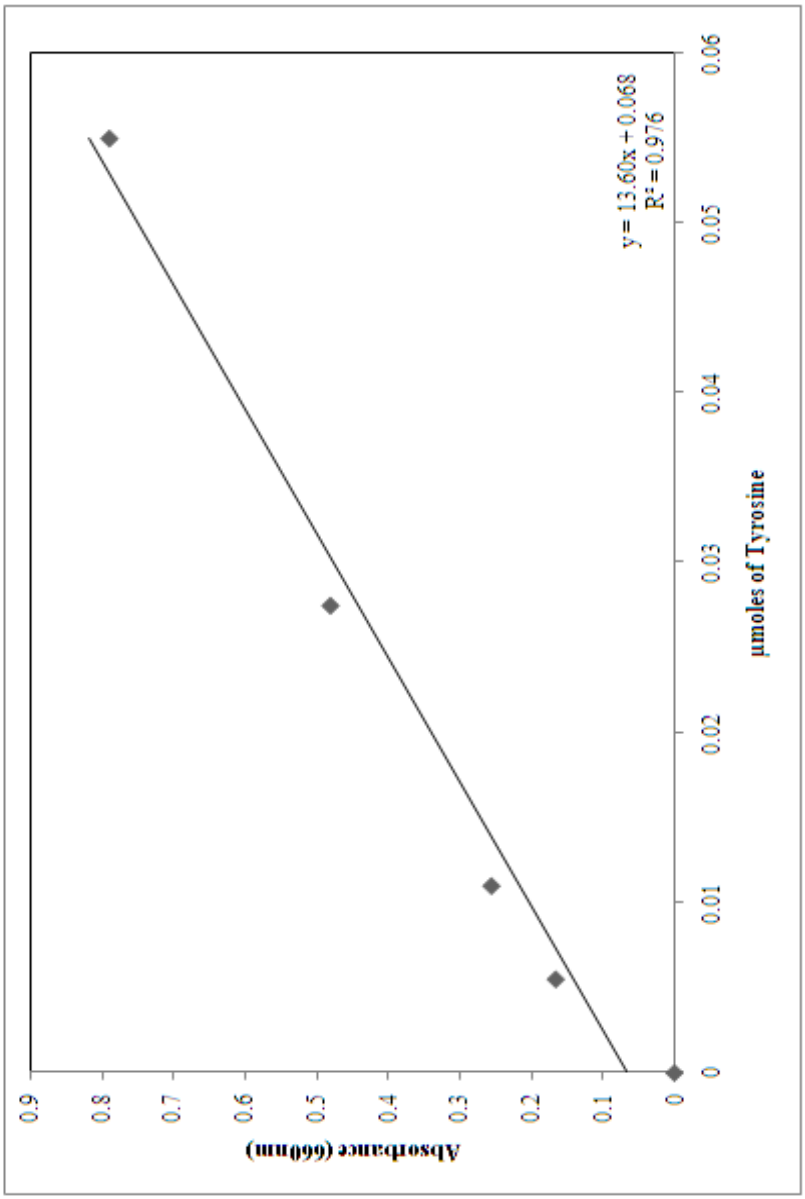
Universal Protein Resources. (2011). *Aspergillus niger*. In *Uniprot (Taxonomy)*. Retrieved on September 27, 2011 from http://www.uniprot.org/taxonomy/5061.

Varnalis, A. I., Brennan, J. G., MacDougall, D. B., and Gilmour, S. G (2004). Optimization of high temperature puffing of potato cubes using response surface methodology. *Journal of Food Engineering.* 61: 153 – 163.

Zambare, V. (2010). Solid state fermentation of *Aspergillus oryzae* for glucoamylase production on agro residues. *International Journal of Life Sciences*. 4: 16-25.

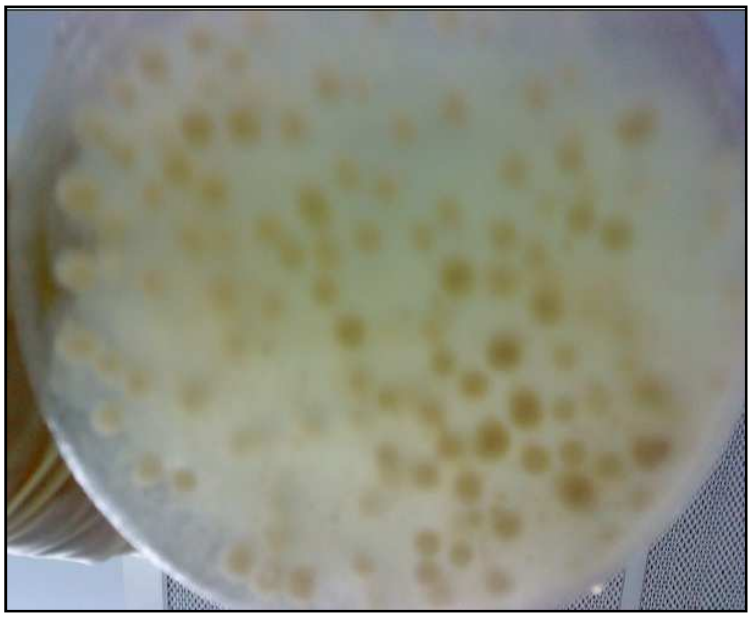
APPENDIX A

TYROSINE STANDARD CURVE

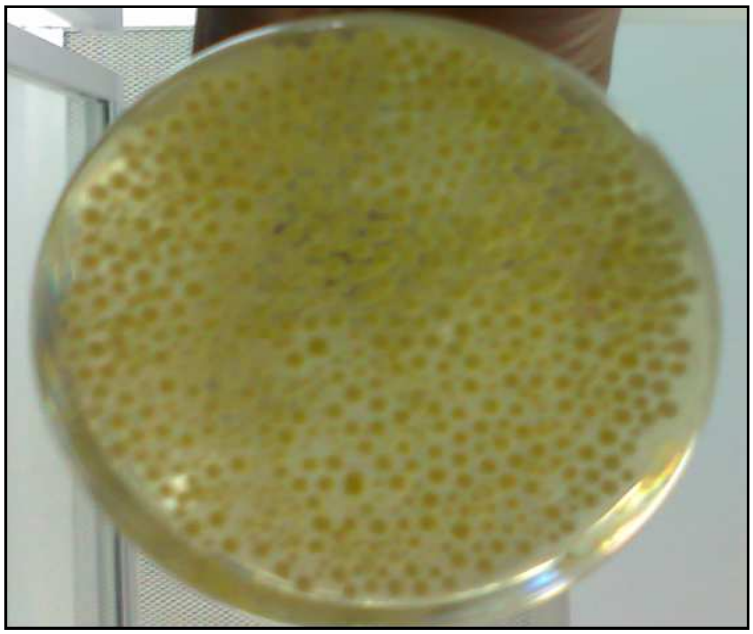


APPENDIX B

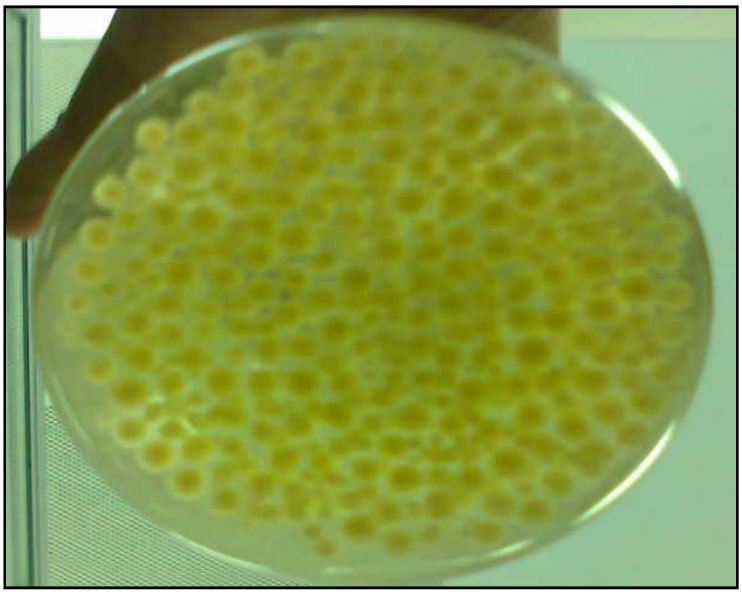
EFFECT OF AGITATION SPEED



At 368 rpm

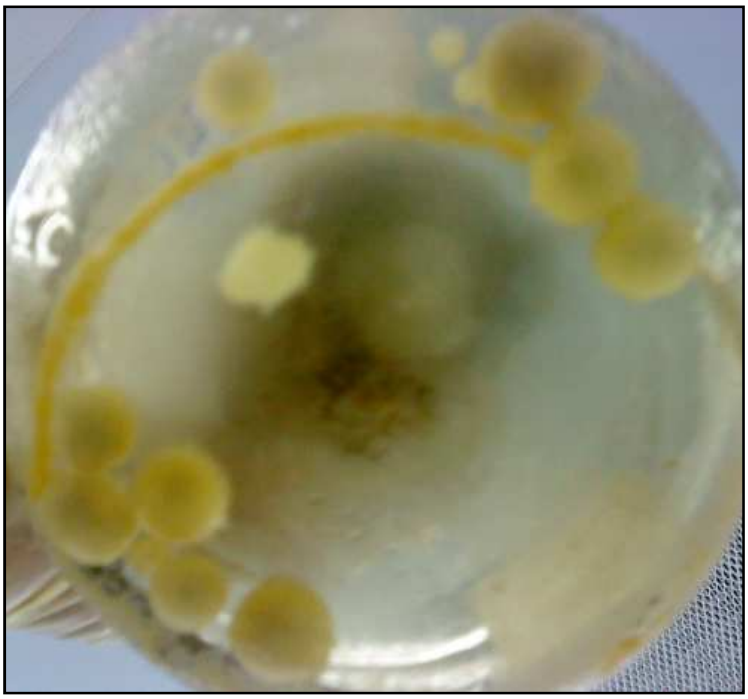


At 300 rpm



At 200 rpm

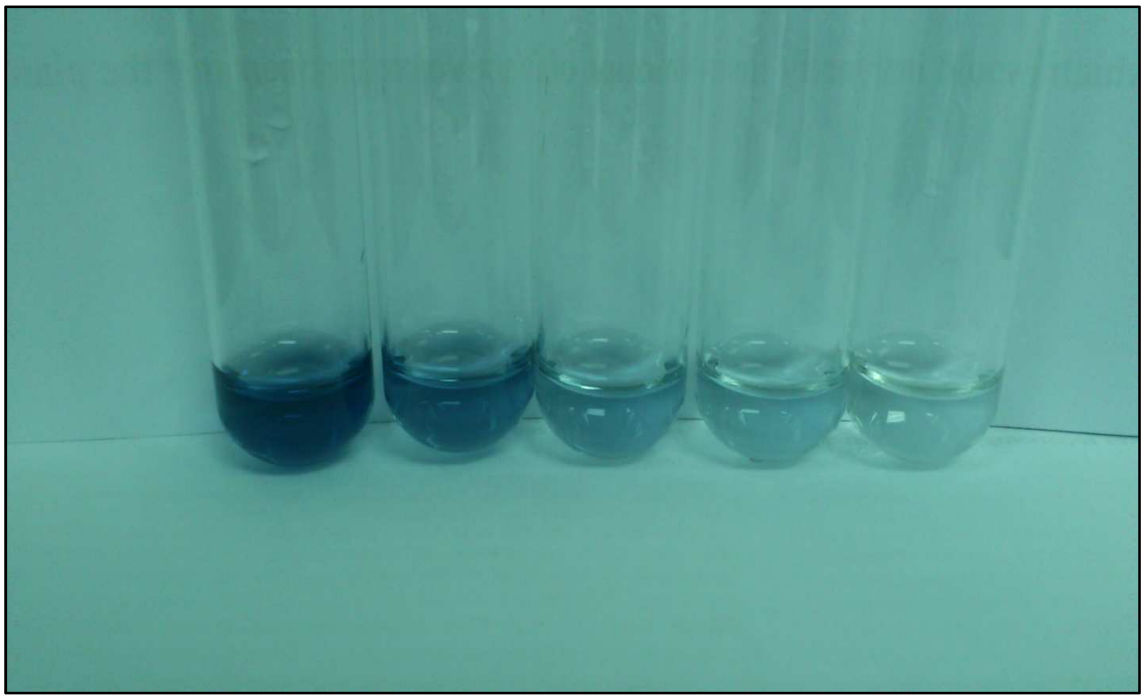
At 100 rpm



At 31 rpm

APPENDIX C

PREPARATION OF TYROSINE STANDARD



APPENDIX D

PROTEASE ASSAY