BORANG PENGESAHAN STATUS TESIS♦

JUDUL : PRODUCTION OF EXTRACELLULAR TANNASE ENYZME BY *ASPERGILLUS NIGER*

SESI PENGAJIAN : 2011/2012

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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.

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ABSTRACT

Tannase catalyses the hydrolysis reaction of the ester bond present in gallic acid esters and hydrosable tannins. This enzyme is produced by plants and microorganism and used in food industry such as tea industry. In order to produce high yields of tannase enzymes, it needs optimization of parameters which normally consuming a lot of time and expensive to be optimized conventionally. In this study, Response Surface Methodology (Central Composite Design) was performed for optimization. *Aspergillus niger* had been chosen as biomass and glucose as substrate in this study. The fermentation study was take place in the shake flask and was optimized for higher tannase enzyme activity. There are three factors was studied in this study which are the pH of the fermentation medium (pH 3.5-pH 6.0), the substrate concentration (2 w/v%-10 w/v%) and the agitation speed (100 rpm-300 rpm). From one factor at a time (OFAT) analysis, tannase enzyme showed that the optimum tannase activity at pH 5, 6.0 w/v% and 200 rpm with 14.9521 U/ml, 13.1256 U/ml and 12.4301 U/ml. RSM results shows that the optimum values of each parameter were pH 4.75, 6.0 v/w% of substrates concentration and 200 rpm of agitation speed which resulted the optimum tannase activity at 15.3131 U/ml. As a conclusion, RSM is the best tool used to identify the correlation between controlled independent factors and observed dependent responses. For the future research, it is recommended for study the application of genetic engineering in the enzyme production by scale up tannase production using a bioreactor and toxicology studies on tannase enzyme for application in food industry.

ABSTRAK

Tannase menjadi pemangkin tindak balas hidrolisis ikatan ester dalam ester asid gallic dan tannic hidrolisis. Enzim ini dihasilkan oleh tumbuh-tumbuhan dan mikroorganisma dan digunakan dalam industri makanan seperti industri teh. Dalam usaha untuk menghasilkan hasil enzim tannase yang tinggi, ia memerlukan pengoptimuman parameter yang biasanya mengambil banyak masa dan mahal untuk dioptimumkan konvensional. Dalam kajian ini, Kaedah Tindak Balas Permukaan (Reka Bentuk Komposit Berpusat) telah dijalankan untuk pengoptimuman. *Aspergillus niger* telah dipilih sebagai biomas dan glukosa sebagai substratu dalam kajian ini. Kajian fermentasi berlaku dalam kelalang goncang dan dioptimumkan untuk aktiviti enzim tannase yang lebih tinggi. Terdapat tiga faktor telah dikaji dalam kajian ini dimana pH untuk medium fermentasi (pH 3.5-pH 6), kepekatan substratu (2 w/v %-10 w/v %) dan kelajuan pengadukan (100 rpm-300 rpm). Dari analysis OFAT, enzim tannase menunjukkan bahawa aktiviti enzim tannase optimum pada pH 5, 6.0 w/v % kepekatan subsratu dan 200 rpm kelajuan pengadukan dengan 14.9521 U/ml, 13.2156 U/.ml dan 12.4301 U/ml. RSM menunjukkan bahawa nilai-nilai optimum parameter setiap pH 4.75, 6.0 w/v% kepekatan substratu dan 200 rpm kelajuan pengadukan yang mengakibatkan aktiviti tannase optimum pada 15.3131 U/ml. Sebagai kesimpulan, RSM adalah alat terbaik yang digunakan untuk mengenalpasti hubung kait antara factor bebas yang dikawal dan diperhatikan keputusan bergantung. Untuk penyelidikan pada masa akan dating, adalah disarankan untuk mengkaji aplikasi kejuruteraan genetik dalam penghasilan enzim, meningkatkan skala pengeluaran tannase dengan menggunakan bioreaktor dan kajian toksikologi enzim tannase untuk aplikasi dalam industry makanan.

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%	Percentage
$+\infty$	High factorial point
^0C	Degree Celsius
$-\infty$	Low level of factorial point
b_{ij}	Cross product coefficient
X	Cell dry weight
X_1	pH of samples
X_2	Substrate concentration of samples
X_3	Agitation speed of samples
Y	Tannase activity
ε	Error

LIST OF ABBREVIATIONS

% w/v	Percentage of weight over volume
$(NH_4)_3PO_4$	Ammonium Phosphate
Aa20	Strain number of *Aspergillus niger*
Adj R^2	Adjusted R^2
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CCD	Central Composite Design
cm	Centimeter
DOE	Design of Experiment
et al.	An others
Exp	Experiment
$FeCl_3$	Ferum chloride
g	Gram
HCl	Hydrochloric Acid
Inc.	Incorporate
KH_2PO_4	Kalium dihydrogen phospate
M	Molar unit concentration
MARDI	Malaysian Agricultural Research and Development Institute
$MgSO_4.7H_2O$	Magnesium Sulphate Anhydrose
ml	milliliter
mL/min	Milliliter per minute
mm	Millimeter
NaCl	Sodium Chloride

NaOH	Sodium Hydroxides
OFAT	One factor at a Time
PDA	Potato Dextrose Agar
rpm	Revolution per minute
RSM	Response Surface Methodology
sp	Species
U	Unit of enzyme
U/g	Units of enzyme per gram
U/L	Units of enzyme per liter
U/L.h	Units of enzyme per liter per hour
Ver	Version
$ZnSO_4$	Zinc Sulfate

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Enzyme is proteins that occur in nature and increase the rate of the biochemical process. In the food industries, enzyme is used to produce everything like cheese to baked goods. This tannase enzyme application is used for the industry because it allows the manufacturer to produce more of a particular product in a shorter amount of time thus increasing the profit.

Nowadays, food industry represents one of the economic sectors by using the microbial in their application. In the field of biotechnology there are many industrial applications use to produce the biotechnological product that we use every day. Most of the enzyme has been produced by submerged culture at industrial level. Some of the food industries apply the enzyme to produce or make improvements in the quality of different foods. The microbial enzyme used as an aid processing in food industries (Belmares *et al.*, 2004)

Employment of the enzymes in food industry are the control of quality of certain foods , the modifications of the properties of some food additives and of the food itself and the production of enzymes used for food additives. In the wine industries, the enzymes are used for increasing the processing capacity and improve the economy (Belur *et al*., 2010)

1.2 PROBLEM STATEMENT

Nowadays, the tannase enzyme is important to the food industry due to high demand in food and drink industry. There are many research had been done to develop the production of the extracellular tannase enzyme. But the production of the tannase enzyme is very low. Because of that, there are needs for an improved process for the production of tannase enzyme.

The economy problem was the major problem for the industry. The price for the enzyme is too expensive. So, by the ability from the microorganism to produce the enzyme, it can solve the economic problem to the industry.

There are a lot of microorganism can produce enzyme. So, the type of the microorganism is decided to be used in this study has become serious matter to observed. Because of that, after some review, it is decided to use the *Aspergillus sp.* from fungi because of their criteria to produce a lot of enzyme. It also easy to handle compared to bacteria. By addition of substrate like glucose to the culture medium at initial concentration, it will improve the production of enzyme tannase by *Aspergillus sp.*

For addition, the time is also being the problem in this study. Because of that, Response Surface Methodology is used in this study as it saves a lot of time. Moreover, from this method the optimum condition for enzyme production can be achieved.

1.3 OBJECTIVE

The objectives of this study are;

i. To produce extracellular tannase enzyme from the fermentation of *Aspergillus niger* using pure glucose.

1.4 SCOPE OF STUDY

To achieve the objectives, three scopes have been identified in this research:

i. To study the production of extracellular tannase enzyme in shakes flask fermentation using pure glucose as substrate for *Aspergillus niger.*
ii. To study the effects of pH controlling (3.5 – 6), the effects substrate concentration controlling (2 w/v% – 10 w/v%) and effects of agitation speed (100rpm – 300 rpm) to the extracellular tannase production in shake flask fermentation.
iii. To applied Response Surface Methodology using Design of Experimental software to design experimental work for extracellular tannase production.

1.5 RATIONALE AND SIGNIFICANCE

The aim of this study was to estimate optimum parameters values to get higher yield of extracellular tannase production in shake flask fermentation method by pure glucose as the media to *Aspergillus niger* culture.

In this study, the Response Surface Methodology (RSM), a central composite design of experiment is used to obtain the optimum parameter such as sucrose concentration, pH and agitation rate. This method is very important to the future study because it will save a lot of time during do the experiment. Besides, it also developed an equation to get the optimum for the production of the enzyme. Thus, Response Surface Methodology is more suitable and gives an advantage compare to the using one factor at a time.

As an addition, in this study, an extracellular tannase has been choosing. By this methodology, experimental works will easier compare to intracellular that are more costing, consumed a lot of time and tedious. Due to this reason, the extracellular is suitable in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

A research had been done to identify cultivating conditions that influence tannase production by *Aspergillus sp.* The keywords that had been search function of tannase, microorganism that use to produce tannase, production of tannase, parameter used, and methods used to analysis tannase. This research is to find the best method, and parameter to increase the production of tannase.

2.2 TANNASE

Tannin acyl hydrolase or tannase catalyses the hydrolysis reaction of the ester bonds present in the hydrolysable tannins and gallic acid esters. Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight depending on the bonds possessed with protein and polysaccharides (cellulose and protein) (Costa *et al.*, 2008). This enzyme is produced by plants part (bark, needles, heartwood, grasses, seeds, and flowers) and microorganism *(Aspergillus, Bacillus)*. The tannase enzyme catalysed the hydrolysis reaction of the ester bond present in the hydrolysable tannins such as tannic acid, methygallate, ethyl gallate, n-propylgallate, isomygallate, and releasing glucose and gallic acid. The tannase is mainly present in microorganisms *(Aspergillus, Bacillus)* even though it also present in plant and animal (Ayed and Hamdi, 2002; Nishitani and Osawa, 2003). Tannins are widespread in the plant kingdom, and found at different part at vascular plant such as bark, flowers, seeds and grasses (Belmares *et al.*, 2004).

Tannin are group of complex oligomeric chains substance that characterized by the presence of polyphenolic compounds. One of the major characteristic of the tannins is its ability to form strong complexes with protein and other macromolecules such as starch and cellulose and it also cause a reduction in nutrional value of food. There are two groups of tannins, hydrosable and condensed tannins (Lekha and Lonsane, 1997). A typical reaction of the tannase enzyme is hydrolytic cleavage of (-) epigallocatechin-3-ol gallate (Figure 2.1) (Bajpai and Patil, 1997; Lekha and Lonsane, 1997). The hydrolysable tannins are constituted by several molecules of organic, such as gallic, ellagic, digallic and chebullicacis, esterified to a molecule of glucose (Belmares *et al.*, 2004). Molecules with a core of quinic acid instead of glucose have been also considered as hydrolysable tannins, (Figure 2.1) presents some examples of hydrolysable tannins (Mueller-Harvey, 2001). In order to maintain their binding capacity, gallotannin or tannins must have more than two gallic acid constituent esterified to the glucose core. Hydrolysable tannins can be easier to hydrolysed under mild acid or alkaline conditions, with hot water or enzymatically (Lopez-Rios, 1984). Condensed tannis or Proanthocyanidins (Figure 2.2) are complex compounds that considered not being hydrolysable (Belmares *et al*., 2004). Their major constituted are cyaniding and delphinidin which responsible for astringent taste of fruit and wines (Sanchez, 2008). As mentioned before, tannase catalyzed the breakdown of hydrosable tannins such as tannic acid, methygallate, ethyl gallate, *n*-propylgallate and isoamygallate. A Figure 2.3 shows a typical reactions of tannase is the hydrolytic cleavage of (-) epigallocatechin-3-ol gallate (Bajpal and Patil, 1997; Lekha and Lonsane, 1997; Belmares *et al*., 2004).

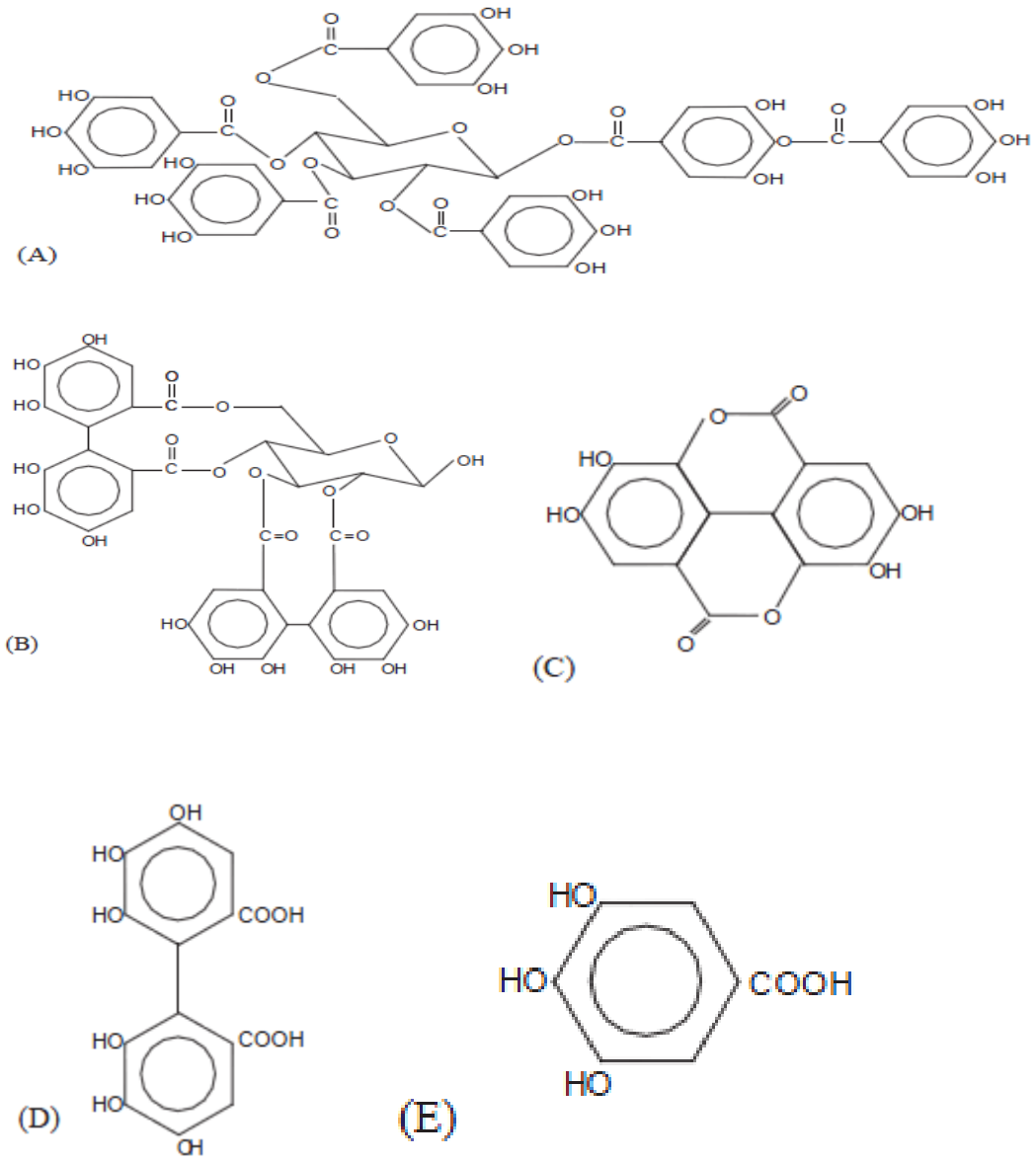

Figure 2.1: Hydrolysable tannins and some of their constituent, A. Gallotannin, B. Ellagitannin, C. Ellagic acid, D. Hexahydoxyphenic acid and E. Gallic acid.

Source: Mueller-Harvey, 2001

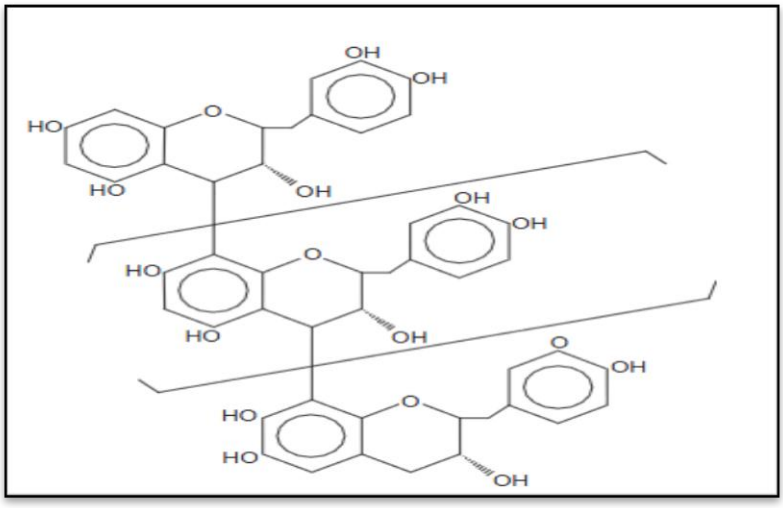

Figure 2.2: Condensed tannins or Proanthocyanidis.

Source: Belmares *et al.*, 2004

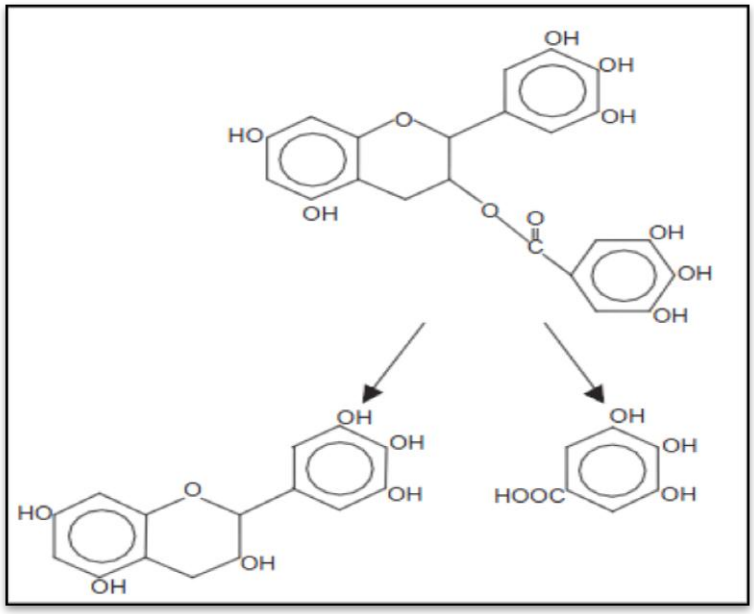

Figure 2.3: Typical reaction of tannase enzyme

Source: Belmares *et al.*, 2004

2.3 THE APPLICATION OF TANNASE ENZYME

Nowadays, the enzymes are commonly used in the industry such as chemical industry, food industry and many more according to their reactions. The advantages of using the enzyme are the mild reaction conditions, lower risk of toxic by-product, and their specificity (Aguilar *et al*., 2001). In the food manufacture, enzymes are mostly used as processing aids to improve the yield, texture, taste or other quality aspects. Enzyme tannase has also been expanding its uses in the global industry (Belmares *et al.*, 2004).

Other important application of tannase in the food industry is its uses as substrate for the chemical synthesis of pyrogallol or ester gallates, which are used as preservatives (Sharma and Gupta, 2003). Recently, tannase enzyme was commercialized in the hydrolysis of gallotannin to gallic acid that is important for the synthesis of propyl gallate in the food industry and it is an intermediate required for the synthesis of an anti-folic antibacterial drug trimethoprim. Gallic acid is extensively used as an ingredient of developer in photography and printing inks. As antioxidant gallic acid acts an anti-apoptotic agent and helps human cells against oxidative damage. Gallic acid also shows their cytotoxic activity against cancer cells, without harming normal cells (Bajpai and Patil, 2008).

This enzyme is extensively used in the food industry as clarifying agent such as preparation of instant tea, coffee, flavored soft drinks and also as additive for detannification of food (Lokeswari and Jaya Raju, 2007).

2.4 MICROORGANISM USED FOR TANNASE PRODUCTION

There are many microorganisms used for tannase production. Most of reported tannase production organisms are fungi and only a few bacteria (Lokeswari, 2010). For example, for bacteria are *Bacillus pumilus, Bacillus polymyxa, Corynebacterium sp, Klebsiella pneumonia, Streptococcus bovis,* and *Selenomonas remunantium,* for yeast are *Candida sp, Mycotorula japonica* and for fungi are *Aspergillus sp., Penicillium*

chrysegenum, Rhizopus oryzae, Trichoderma viride, Fusarium solani and *Mucor sp.* The production of tannase is depending on the strain and the culture conditions. It is showing different patterns for the different microorganisms. The filamentous fungi of the *Aspergillus* genus have been widely used for tannase production (Bajpal and Patil, 1996, Banerjee *et al*., 2001).

The production of the tannase enzyme by the *Aspergillus sp.* can occur in the absence of tannic acid, but these fungi tolerate tannic acid concentrations as high as 20% without having a deleterious effect on both the growth and enzyme production.The main advantage of producing fungi compared to bacterial cells is that they are typically much larger and are easily separated from the fermentation medium. (Belmares *et al.*, 2004)

Fungi also grow much more slowly than bacteria. A slower growth means that less nucleic acid is contained in the end product. In additions, fungi also have lysine content and ability to grow at acid pH. The disadvantages include lower growth rate, lower protein content and lower methionine content than in bacteria. For this research, the tannase produced by *Aspergillus niger* that have been carried out on submerged and solid state cultures. The addition of the carbon sources such as glucose to the culture medium, it will improve the production of tannase by *Aspergillus niger.* Table 2.1 show the microorganisms used for tannase production (Belmares *et al*., 2004).

Table 2.1: Microorganisms used for tannase production

Types of Microorganisms	
Bacteria	*Bacillus pumilus*
	Bacillus polymyxa
	Corynebacterium sp.
	Klebsiella pneumoniae.
	Streptococcus bovis
	Selenomonas rumunantium
Yeast	*Candida sp.*
	Saccharomyces cerevisiae.
	Mycotorula japonica
Fungi	*Aspergillus niger*
	Aspergillus oryzae
	Aspergillus japonicas
	Aspergillus gallonyces
	Aspergillus awamori
	Penicillum chrysogenum
	Rhizopus oryzae.
	Trichoderma viride
	Fusarium solani
	Mucor sp.

Source: Belmares *et al.*, 2004

2.5 *ASPERGILLUS NIGER*

Aspergillus niger in of the filamentous fungi that have been widely used for tannase production (Belmares *et al.*, 2004*). Aspergillus niger* is a filamentous fungus that give an important role in biotechnology. Table 2.2 shows the taxonomy of *Aspergillus niger.*

Table 2.2: Taxonomy of *Aspergillus niger*

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

Sources: Universal Protein Resources, 2011

Aspergillus niger does have spores and reproduces asexually meaning that it can produce its offspring by themselves. According to Purwanto *et al.* (2009) research, they discovered that the morphology of *Aspergillus niger* using Scanning Electron Microscope (SEM). Meanwhile Rob Hoffmann (2010) identified the SEM of the asexual reproduction of *Aspergillus niger*.

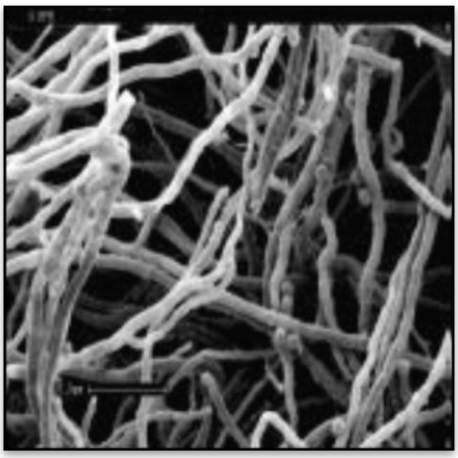

Figure 2.4: Filamentous structure of *Aspergillus niger*

Source: Purwanto *et al.*, 2009

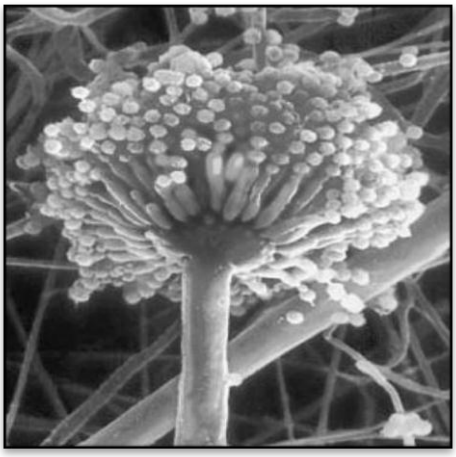

Figure 2.5: Asexual reproduction of *Aspergillus niger*
Source: Rob Hoffman, 2010

Filamentous fungi must have potential to grow under varying environmental conditions such fermentation time, pH, temperature and utilizing various sources of substrate such as nutrients (Ikram-Ul-Haq *et al.,* 2006). The tannase production can be produced by *Aspergillus niger* (Lokeswari and Jaya Raju, 2007), *Aspergillus oryzae* (Lokeswari, 2010), *Aspergillus tamari* (Costa *et al.,* 2008), and *Aspergillus awamori* (Beniwal and Chhokar, 2010).

2.6 PRODUCTION OF TANNASE ENZYME

The tannase enzyme production from *Aspergillus* can occur in absence of tannic acid, this fungi tolerates tannic acid concentration as high 20 percent without having a deleterious effect on both growth and enzyme production (Belmares *et al.,* 2004). The studies on tannase enzyme production by *Aspergillus sp.* can be done in various methods. There are various ways to produce the tannase from the microorganisms; they are liquid surface, submerging culture, modified solid-state cultures and solid state culture (Bradoo *et al.,* 1997, Belmares *et al.,* 2004).

The term of solid state fermentation (SFF) is applied for the processes in which insoluble material in water are used for the materials in water are used for the microbial growth (Aguilar *et al*., 2008). However, solid state fermentation is used to produce more than one enzyme and it variety, mainly from mold origin. This fermentative process

needs the water that does not exceed the capacity of the saturation of the solid bed in which the microbial grow. Water is essential for the microbial growth in the solid state fermentation and it present in thin layer and in occasions, absorbed inside these substrates (Aguilar *et al*., 2008).

The production of tannase by solid state culture has more advantage than submerged culture. The advantages of the solid state culture are it has the high production titles more than submerged culture, the extracellular nature of the enzymes and the stability to wide pH and temperature ranges (Lekha and Lonsane, 1994). Higher enzyme activities have been reached using solid state culture. The tannase activity also has maximum expressed intracellular in solid state culture 18 times more than submerge culture, while the extracellular activity in the solid state culture is 2.5 times more than submerge cultures. The solid state culture system minimizes the catabolic repression phenomenon. However, most of enzyme manufacture produces the enzyme using submerged culture or liquid surface fermentation techniques with enzyme filter of grams per liter (Belmares *et al*., 2004).

For submerged culture, some studies show that optimum production and regulatory aspects of tannase by moulds carried out in submerged culture. There are two major different are found about submerged culture and the solid state culture. That are the tannase yield production and productivity are higher in solid state culture than the submerged culture and the tannase location under solid state culture conditions in mostly extracellular, whilst it is bounded to the mycelium under the submerge conditions (Belmares *et al*., 2004).

From the research done by Aguilar *et al.* (2008) shows that the solid state culture is produce a lot of biomass yield compare to the submerge culture. The tolerance to high concentration of tannic acid by *Aspergillus niger* was lower in the submerge culture than in solid state culture. There are comparison between solid state culture and submerge culture. For solid state culture, the culture media are simple. Some substrate can be used directly as a solid media or enriched it with nutrient. Then, the products of interest are concentrated, that which facilitates its purification.

The quantity of waste generated is smaller than submerged culture. Then, it has low humidity content. The disadvantage of using solid state culture is that the microorganism growth was limited by levels of humidity. The determination of parameters such as humidity, pH, free oxygen and dioxide carbon, constitute a problem due to the lack of monitoring devices. And the scale up of solid state fermentation processes has been little studied and it presents several problems (Belmares *et al.*, 2004)

Different researcher use liquid surface fermentation, submerged fermentation and solid state fermentation for the tannase production. Among these, submerged fermentation process for tannase production is mostly preferred because the sterilization and process-control method are easier to engineer in this system (Paranthaman and Vidyalakshmi, 2009). Most of the tannase enzyme has been produced by submerged fermentation. Its production at industrial level is in a microbial way using submerged culture, where the activity is expressed mainly of intracellular form, implying additional costs in its production (Lekha and Lonsane, 1994). Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns (Belmares *et al.*, 2004).

2.7 FACTOR EFFECTING THE PRODUCTION OF TANNASE ENZYME

According to Ikram-Ul-Haq *et al.* (2006), there are several conditions for the fungi to grow such as fermentation time, pH, temperature and utilizing various sources of substrate as nutrients. So, the effect of the parameters gives an influence to their tannase production.

2.7.1 Effect of pH

Tannase activity and stability are at pH 5 to 6.0 and 3.5 to 8.0 (Belmares *et al.*, 2004). According to Natarajan and Rajendran (2009), the tannase enzyme was active at acidic pH and activity decreased as the pH approached to the alkaline pH range. From their research, the tannase enzyme needs an acidic environment to be active in the case of produced by *Lactobacillus plantarum* strain. The tannase enzyme is known as acidic protein with optimum pH at pH 6 (Belmares *et al*., 2004; Lokeswari, 2010; Mahapatra *et al*. 2005). The effect of pH on the enzyme activity is determined by the nature of the amino acid at the active site, which undergoes protonation and deprotonation and by the conformational changes induced by ionization of the amino acids. Mahapatra *et al.* (2005) stated that any change in pH affects the protein structure and decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability.

From previous studied by Lokeswari and Jaya Raju (2007), the optimum tannase production was recorded at pH 5.5. This proved that the *Aspergillus niger* needed an acidic environment to be active. Beverini and Metche (1990) showed that tannase activity was stable at pH 3.5 and 5.5.

2.7.2 Effect of Agitation Speed

Agitation speed is a very important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium (Purwanto *et al*., 2009). According to Darah *et al*. (1996), the maximum lignin peroxidase activity and a maximum fungal growth were achieved when the optimal agitation speed of 150 rpm was used. They also mentioned that the excessive agitation would produce greater mechanical forces or hydrodynamic shear stresses and this condition is known to damage fungal mycelia and pellets. From previous research by Purwanto *et al.* (2009), at higher agitation rates, the enzyme production dropped. They also stated that, the speed of the culture broth give a variety of effects on microorganism, including rupture of the cell wall, change in morphology of filamentous microorganism, variation in the efficiency and rate of growth and also variation in the rate of formation of the desired product. The result from Natarajan and Rajendran (2009) shows that, the increase in

agitation rate beyond 200 rpm resulted in a drastic fall in specific enzyme activity. However, the best fungal growth achieved when the culture agitated at 100 rpm.

During submerged growth, many filamentous fungi may grow either as free mycelia or as pellets and the growth form is determined by a number of factors such as growth medium, size of inoculum and physical environment (Purwanto *et al.*, 2009). The fungal hyphae or mycelia are influenced by mechanical forces or shear stresses. Figure 2.6 shows the morphological changes of *Aspergillus niger* at static, 0 rpm and 150 rpm.

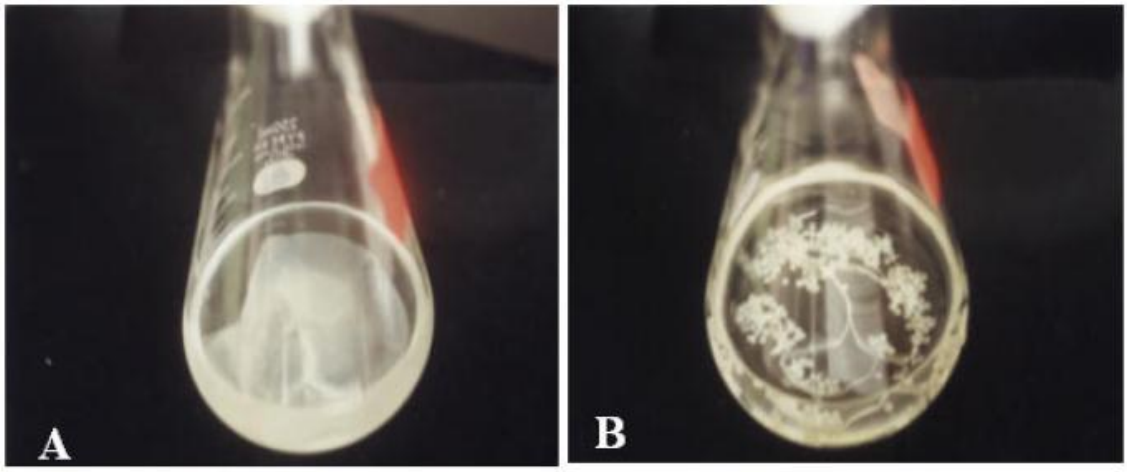

Figure 2.6: The morphological changes of *Aspergillus niger*. (A) Static (0 rpm) and (B) 150 rpm.

Sources: Purwanto *et al.*, (2009)

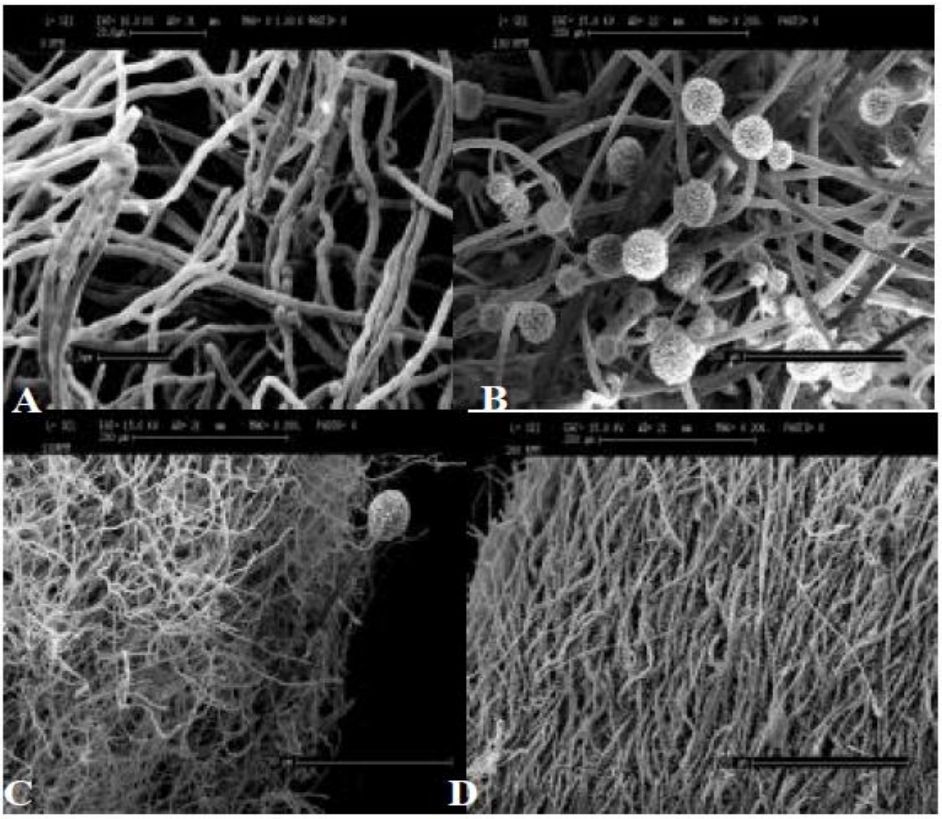

Figure 2.7: SEM micrograph of the effects of various agitation speed on the mycelia of *Aspergillus niger*. (A) 0 rpm, (B) 100rpm, (C) 130 rpm, and (D) 200rpm. Sources: Purwanto *et al*., (2009)

From the previous research by Purwanto *et al.* (2009), the optimal agitation speed was 130 rpm which produced a maximal tannase activity. The agitation speed that has higher than 130 rpm resulted in low enzyme production and this condition could be due to the fungal cell disturbances causes by shear stress or shear forces. Meanwhile, the lower agitation speed less than 130 rpm resulted in low growth, which is resulted in low tannase enzyme. This could be due to low amount of dissolved oxygen in the cultivation medium. The other resulted from that research shows that the fungal pellets to decrease in size but to increase in number per unit volume. At 0 rpm, the fungal mycelium developed a layer of fungal mat (Figure 2.6A) and as the agitation speed increased the fungal pellet formed (Figure 2.6B). The increase in agitation speed was result the higher shear stress causing the pellets of smaller size and dense.

Based on the Figure 2.7, it shows the scanning electron microscope of the effect of various agitation speed of on the mycelia of *Aspergillus niger*. It was found that at 0 rpm or static, the fungal hyphae were cylindrical and loose in shape. When the agitation speed increased, the hyphae became thinner and denser (Figure 2.7B), with less fruiting body (Figure 2.7C). At 200 rpm (Figure 2.7D) only the tight packing mycelia seen, with the presence of many spikes on them (Purwanto *et al*., 2009).

2.7.3 Effect of Substrate Concentration

Lekha and Lonsane (1997) reported that the initial tannic acid concentration affected the levels of enzyme. As mention earlier, the production of tannase by *Aspergillus* can occur in the absence of tannic acid, this fungi tolerates tannic acid concentration as high as 20 % without having a deleterious effect on growth and enzyme production. By addition of carbon sources such as glucose, fructose, sucrose, maltose, arabinose to the culture medium at initial concentration from 10 to 30 g/l improves tannase production by *Aspergillus niger* (Belmares *et al*., 2004). From Lokeswari and Jaya Raju (2007) research, they mentioned that glucose at higher concentration repressed tannase synthesis while the lower concentration was not repressive. As pointed out by Beverini and Metche (1990), the lower concentration of glucose is not repressive for enzyme production in *Aspergillus japonicas* but its concentration above 1.0 % is inhibitory for both growth and enzyme production. The synthesis of tannase is not under catabolic repression in the presence of glucose or sucrose (Aguilar *et al*., 2001). Glucose is involved on catabolite repression and gallic acid may be involved in end-product repression. The addition of glucose in the submerged cultures of *Aspergillus japonicas* using tannic acid as substrate did no decrease the production of tannase (Bradoo *et al*., 1997).

The ability of *Aspergillus tamari* to grow and to produce tannase on different carbon was studied by Costa *et al.* (2008). From their study, the high productions of tannase activity were obtained by gallic acid, tannic acid and methyl gallate. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid have been described as tannase inducers (Bajpai and Patil, 1997). Costa *et al*. (2008) also mentioned that addition of higher amount of glucose drastically reduced the production

of the enzyme by using gallic acid as inducer and glucose concentration as potential repressor. According to Kumar *et al.* (2007), it may be due to the fact that the substrate is already rich enough to supply the nutrients required for fungal growth and tannase production. From the previous studied by Sabu *et al.* (2006), the tannase production was inhibited by the incorporation of any of the carbon sources to the different substrates except tamarind seed powder.

2.8 OPTIMIZATION OF TANNASE PRODUCTION

Table 2.6: Optimization of several parameters with their optimum tannase activity

Species	State	Studied parameter	Value	Optimum Tannase Activity(U/ml)	Reference
A.niger	SmF	Glucose conc. (%, w/v)	0.01	16.61	Lokeswari and Jaya Raju (2007)
			0.05	19.22	
			0.10	20.32	
			0.50	21.42	
			1.00	18.32	
			1.50	17.24	
		pH	3.5	14.32	
			4.0	16.28	
			4.5	18.64	
			5.0	20.02	
			5.5	22.62	
			6.0	16.42	
A. flavus	SmF	Tannic acid conc. (%)	1.0	18	Paranthaman *et al.* (2009)
			1.5	26	
			2.0	44	
			2.5	28	
			3.0	23	
		Time (h)	24	18	
			48	29	
			72	32	
			96	39	

Table 2.6: Continued

Species	State	Studied parameter	Value	Optimum Tannase Activity (U/ml)	Reference
A.niger	SmF	Agitation Speed (rpm)	0	1.75	Purwanto *et al.*(2009)
			100	1.98	
			130	2.67	
			150	2.54	
			200	1.45	
A. oryzae	SmF	pH	3.5	24.32	Lokeswari (2010)
			4.0	28.94	
			4.5	30.56	
			5.0	32.62	
			5.5	29.62	
			6.0	28.62	
A.niger	SmF	Glucose Concentration (g/l)	6.25	0.083	Aguilar *et al*. (2001)
			12.5	0.161	
			25.0	0.099	
			50.0	0.042	

2.9 RESPONSE SURFACE METHODOLGY

In biotechnology field, optimization of culture condition through Response Surface Methodology (RSM) and Fractional Factorial Design (FFD) is a common practice. It 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 are many parameters at a time to be studied in every fermentation process. According to Saravanakumar *et al*. (2010), studying one parameter at a time while holding the other parameters constant is the optimization process that done classically and this will normally takes such a long period of time and quite costly, when large number of variables are evaluated. Bas and Boyaci (2007) mentioned this technique as known as one-factor-at-a-time (OFAT) and it does not consider the effect of interaction of various parameters. It does not represent the complete effects of the parameters. Because of that, Response Surface Methodology (RSM) is important to being introduced and suitable for optimization purpose.

RSM is an empirical modelization method used in the evaluation of the relationship of a set of controlled experimental factors and observed response (Annadurai and Sheeja, 1998). Adinarayana and Ellaiah (2002) stated that RSM is useful model for studying factors that affect the response by varying them simultaneously and it can also be to study the relationships between one or more factors (independent variables) and responses (dependent variables). It also can be applied in both chemical and biochemical processes (Bas and Boyaci, 2007).

According to Bas and Boyaci (2007) while using RSM, the optimization process should undergo three vital stages. The first stage is the preliminary study for estimating the independent parameters to 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 slot and contour plot of the response and finally determining the optimum values.

The interaction between the parameters can be clearly illustrated by response surface 3D plots (Dutta *et al*., 2004) and also contour plots. Bas and Boyaci (2007) said that RSM offers significant number of advantages compared to classical optimization method where RSM provides more information although with small number of experiment and the interaction between parameters can be study. By using the RSM method, it wills reduces the number of trials and provide multiple regression approach (Adinarayana and Ellaiah, 2002; Gan *et al*., 2007).

RSM also have the negative part where the data needs to fit second order polynomial but all systems can be represent by second order polynomial (Bas and Boyaci, 2007). This problem can be solved 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 the independent parameters. It also cannot be used for all 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 (Bas and Boyaci, 2007). This is because it is only can be used for data that can be explained by second order model and not all systems can be fitted in second order model.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter is discussing the method and material that will be used to conduct a study on the cultivating conditions that influence the tannase production by *Aspergillus niger* in submerged culture. The experimental design was divided in three major sections. The experiment was conduct in a sequence starting from cultivation to medium growth, fermentation of *Aspergillus niger* with different parameters used to produce the tannase according to objectives of this research and analysis of data obtained after the experiment.

3.2 CULTIVATION OF MICROORGANISM

A fungal strain used in the present study was *Aspergillus niger* that obtained from Malaysian Agricultural Research and Development Institute (MARDI), Selangor Darul Ehsan, Malaysia. The *Aspergillus niger* was used as the microorganism in this experiment to produce the tannase enzyme. The *Aspergillus niger* was grows on the agar medium potato dextrose agar (PDA). The microorganism was periodically subculture by dispersing them using sterile inoculating loop onto fresh agar medium. The microbial was grows fast at room temperature (30^{o}C). The slant culture was used for the further work or stored in refrigerator at 4°C.

3.3 INOCULUM PREPARATION

After culture the microorganism, the *Aspergillus niger* spores from the PDA slant were used for inoculums preparation before being used in inoculating in the fermentation medium. 100ml of inoculums broth prepared in the Erlenmeyer flask was composed by 0.1 g yeast extract and 1 g of glucose inoculated with 2% of 1-week-old *Aspergillus niger* strain. Then, it was incubated at 30°C, 250 rpm for 24 hours.

3.4 PREPARATION OF PRODUCTION MEDIA

In this procedure, the experiment will studied by three different parameters with different range selected due to meet the objectives. Since there are three parameters will be used, therefore experiments will be conducted by manipulating one factor with constant the two variables. The enzyme production was continued by the preparation of medium that contain of 1 g/l tannic acid, 1 g/l of yeast extract, 0.005 g/l, 1g/l KH_2PO_4, 5g/l of $(NH_4)_3PO_4$, 0.1 g/l of $ZnSO_4$, 1 g/l of Mg_2SO_4.

3.4.1 The Effect of Substrate Concentration

In order to study the effect of substrate concentration on tannase activity, the glucose concentration is increased from 2 w/v% to 10 w/v%. The difference glucose concentration was added in the production media meanwhile the other parameters was constant at pH 5 and 200 rpm of agitation speed at 30°C for 40 hours. This procedure was repeated by using another glucose concentration in submerged culture.

3.4.2 The Effect of pH

In order to study the effect of pH on tannase activity, the pH of the production media is set from pH 3.5 to pH 6. The pH of the production media was adjusted by 1M of HCl and 1M of NaOH. To achieve this study, the other parameters must be constant at 6 w/v% of glucose concentration and 200 rpm of agitation speed at 30°C for 40 hours. This procedure was repeated by using the different pH in submerged culture.

3.4.3 The Effect of Agitation Speed

To achieve the objective of the study about the effect of agitation speed on tannase activity, the range of agitation speed was set from 100 rpm to 300 rpm. The production media was constant with the other parameters at pH 5 and 6 w/v% of glucose concentration. This experiment was carried out in the incubator shaker at 30°C for 40 hours. This procedure is repeated by used the different of agitation speed in submerged culture.

3.5 EXPERIMENTAL DESIGN AND PROCESS OPTIMIZATION

The statistical analysis for the production of extracellular tannase enzyme by *Aspergillus niger* was performed by using Design Expert Ver 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 tannase activity, certain fermentation conditions such as pH (pH 3.5, 4.75 and 6.0), substrate concentrations (2 g/l, 6 g/l and 10 g/l) and agitation speed (100 rpm, 200 rpm and 300 rpm) were optimized. Each of these variables is varied over five levels which were low axial point (-∞), low factorial point (-1), central point (0), high axial point (+∞) 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 3.2 shows the design matrix of CCD for extracellular protease production. Tannase activity (Y1) and cell dry weight (Y2) were 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 (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad \ldots (1)$$

Where: Y= responses; β_0= constant; β_1,β_2,β_3= linear regression; β_11,β_22,β_33= interaction regression; X_1,X_2,X_3= variables.

Table 3.1: Experimental design of CCD for extracellular tannase production

Run	**X_1 pH**	**X_2 Substrate Concentration (w/v%)**	**X_3 Agitation Speed (rpm)**
1	4.75	6.00	31.82
2	4.75	0.73	200.00
3	6.85	6.00	200.00
4	3.50	10.00	300.00
5	6.00	10.00	100.00
6	6.00	2.00	100.00
7	4.75	12.73	200.00
8	4.75	6.00	200.00
9	4.75	6.00	200.00
10	3.50	10.00	100.00
11	3.50	2.00	100.00
12	6.00	10.00	300.00
13	4.75	6.00	200.00
14	6.00	2.00	300.00
15	4.75	6.00	200.00
16	3.50	2.00	300.00
17	4.75	6.00	200.00
18	4.75	6.00	368.18
19	2.65	6.00	200.00
20	4.75	6.00	200.00

3.6 ENZYME ASSAY

Tannase activity was determined by the method of Mondal and Pati (2000). To assay the enzyme tannase, the enzyme solution (0.1 ml) was incubated with 0.3 ml of 1.0% tannic acid, and in 0.2 M acetate buffer (pH5.0) at 40°C for 30 minutes and then the reaction was terminated at 0°C by the addition of 2 ml BSA (1mg/ml), which the precipitates the remaining tannic acid. A control reaction also done side by side with heat denatured enzyme. The tubes were centrifuged and the precipitate was dissolved in 2 ml of SDS-triethanolamine (1% w/v triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 ml of $FeCl_3$ (0.13 M).

Then, the tannase activity is determined by calculated using Equation 3.1. One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyze 1µ mole of ester linkage of tannic acid in 1 minute at specific condition.

$$\frac{U}{ml} = \frac{\mu mol\ gallic\ acid}{(incubation\ time)(volume\ of\ enzyme)} \tag{3.1}$$

3.7 CELL DRY WEIGHT

For the first method of analysis, the cell dry weight is determined by using 25ml samples (three times repeated) which the fungal mycelia will be filter by using Whatman filter paper no 1 and washed with distilled water and dried overnight at 80 °C in oven .Then, the weight is calculated by using the formula as below:

$$\frac{X\left(\frac{g}{l}\right) = [(Weight\ dry\ filter\ paper + Cell) - (Weight\ dry\ filter\ paper)](g)}{Sample\ volume\ (l)} \tag{3.2}$$

3.8 DETERMINATION OF TANNIC ACID STANDARD CURVE

To determine the tannic acid standard curve, different concentration of tannic acid solution (0 w/v%, 2 w/v%, 4 w/v%, 6 w/v%, 8 w/v% and 10 w/v%) was used. These standard solutions were then analyzed by using UV-Vis Spectrophotometer at 550 nm to obtain the absorbency for calibration that used as a reference for release of tannic acid concentration by tannase activity.

3.9 FLOWCHART OF TANNASE PRODUCTION BY *ASPERGILLUSSP* IN SUBMERGED CULTURE

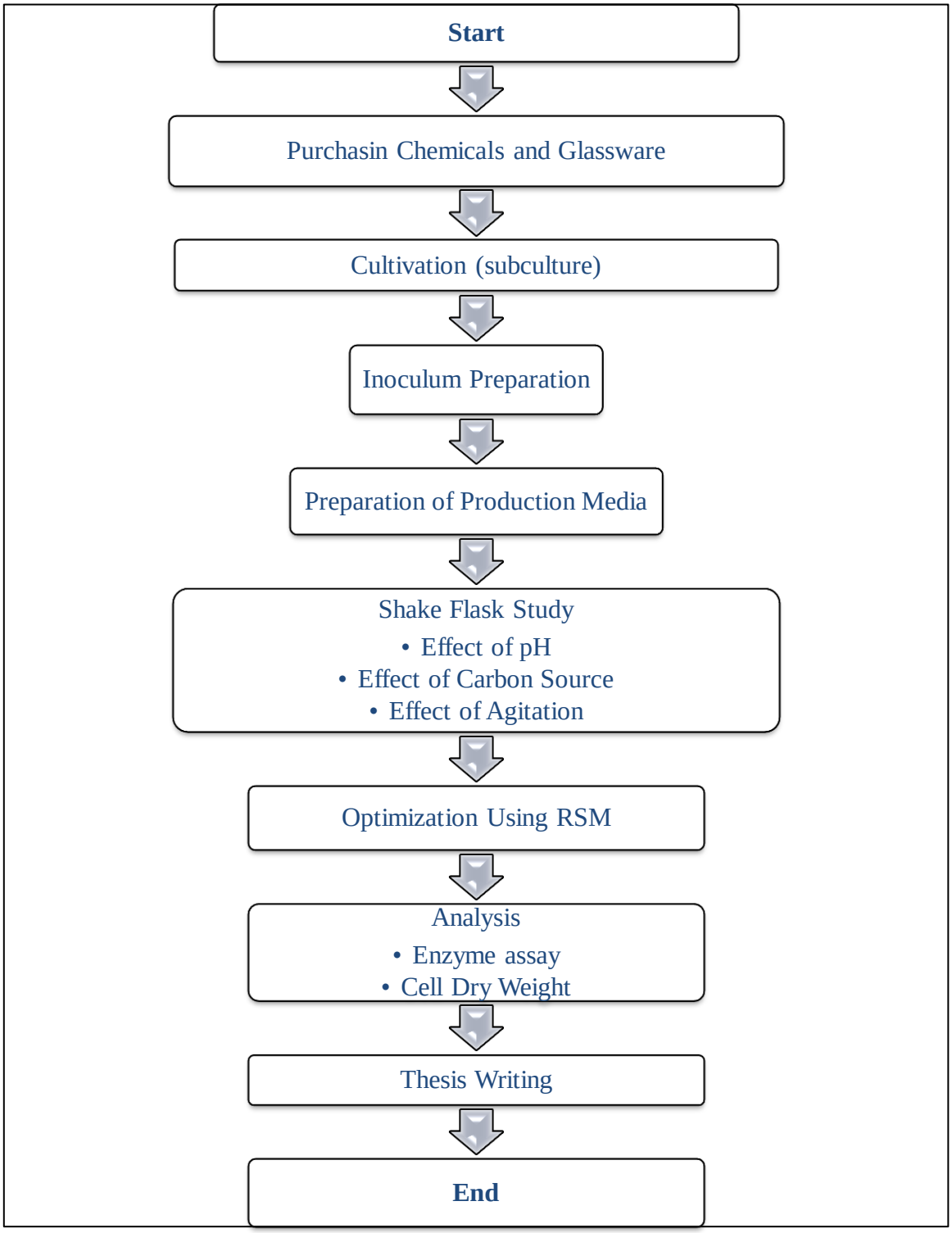

Figure 3.1: Flowchart of Tannase Production

CHAPTER 4

RESULT AND DISCUSSION

4.1 INTRODUCTION

This chapter is discussing the result which is obtained during this study based on the effect of cultivating conditions for each parameters selected on tannase production in submerged fermentation by *Aspergillus niger* which are effect of pH, effect of substrate concentration and effect of agitation speed. The optimization of the tannase activity by using Response Surface Methodology (RSM) also shows in this chapter.

4.2 ONE FACTOR OF A TIME

In shake flask experiments, optimization by using one-factor-at- a- time method was conducted at 30^{o}C for 40 h, which involved changing one independent variable while fixing others at a certain level. The process parameters that involved in this study are pH, substrate concentration and agitation speed. To optimize the tannase production by using a response surface methodology (RSM) couple with central composite design (CCD) was also used in this procedure.

4.2.1 Effects of pH

The production of tannase enzyme is influenced by the pH of the culture medium. To study the effect of pH on the enzyme tannase, substrate concentration and agitation speed is important to keep constant in this experiment.The pH medium was varied from 3.5 to 6.0 using 1M of HCI and 1M NaOH. The enzyme activity was active at acidic pH and activity decreased as the pH approached to the alkaline pH range.

Figure 4.1 shows the effects of pH on enzyme tannase activity. The maximum tannase enzyme activity was observed at pH 5.0 which is showed at 14.9521 U/ml after 40 hours. The graph in Figure 4.1 shows the lowest enzyme activity is at pH 3.5 compared to the other pH where the enzyme activity is only found in 11.9516 U/ml. At pH 4.0 to 4.5, the tannase activities were relatively lower compared to pH 5.0. The tannase activity was significant only when the pH of the medium was in the pH ranges 4.0 to 6.0. At too acidic pH, the enzyme activity was less. From the result, it showed that the tannase enzymes need an acidic environment to be active. This result shows the similarity to the report that published by Lokeswari (2010). Naturally any changes in pH may affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or is instability nature (Natarajan and Rajendran, 2009). According to the report from Belmares *et al.* (2004) it said the properties of the tannase activity are stable at pH 5.0 to 6.0 where it is to be an acidic protein.

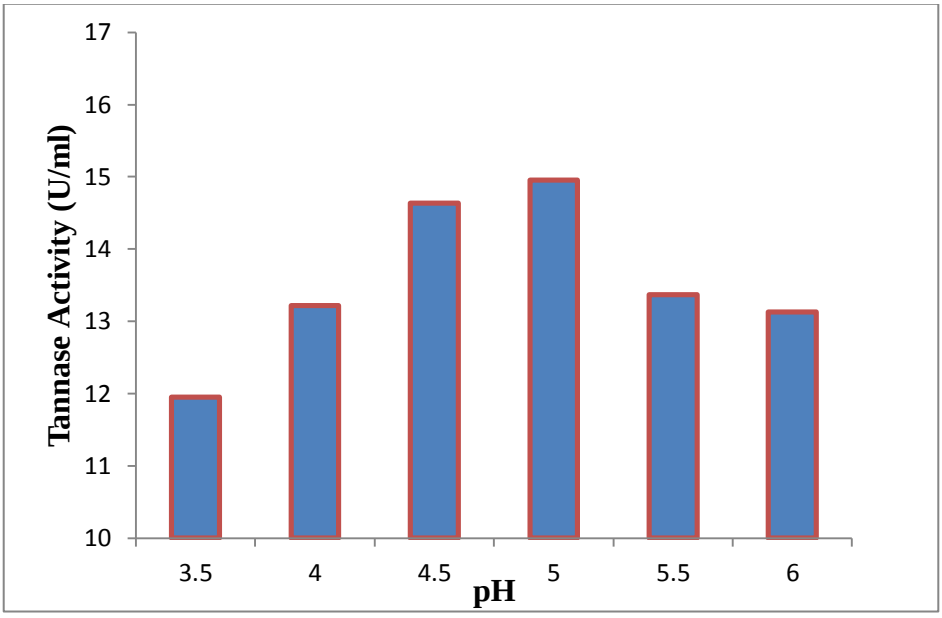

Figure 4.1: Effect of pH on enzyme activities

4.2.2 Effects of Substrate Concentration

The tannase activity also affected by the substrate concentration of the culture medium. To study the effect of different glucose concentration on tannase production, the glucose concentration was varied from 0 w/v% to 10 w/v%. The enzyme activity was found that at higher concentration of glucose repressed the tannase production while lower concentration was not repressive. From Figure 4.2, the maximum enzyme production present at 6 w/v% glucose in the tannic acid medium at 13.1256 U/ml. According to Beverini and Metche (1990), the lower concentration of glucose is not repressive for enzyme production *Aspergillus japonicas* but its concentration above 1.0 w/v% was inhibitory for both growth and enzyme production. From Lokeswari and Jaya raju (2007) research, the maximum production of tannase was at 0.5 w/v% that produced 21.42 U/ml of tannase activity. The tannase activity was reduced after the glucose concentration above 1 w/v%.

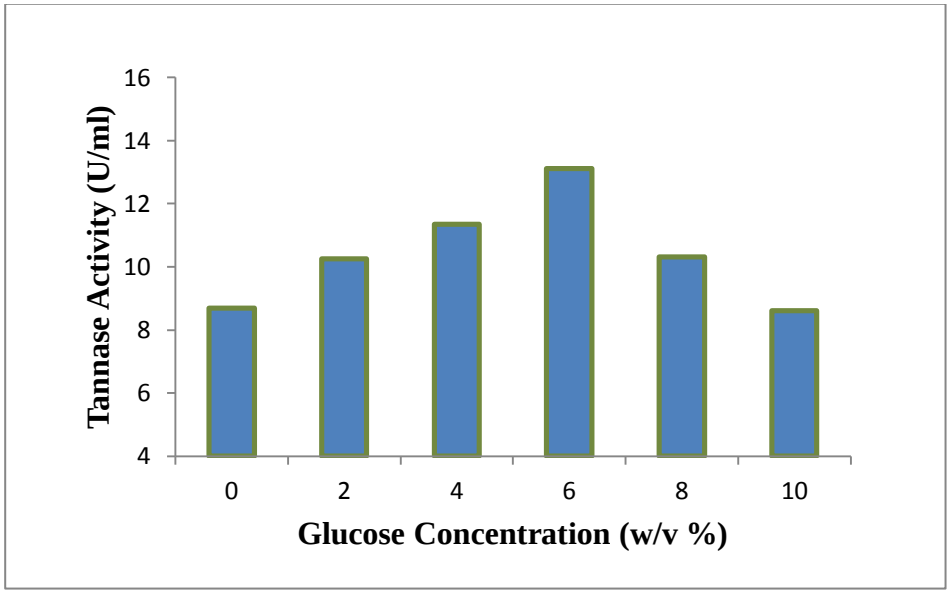

Figure 4.2: Effect of effect of substrate concentration on tannase activity.

4.2.3 Effects of Agitation

To study the effect of agitation speed on tannase activity, the other conditions must be kept constant. The effects of agitation speed on tannase activity by *Aspergillus niger* in submerged fermentation are shown in Figure 4.3. The optimum agitation speed for the maximum tannase activity is at 200 rpm which produced a maximal tannase activity of 13.2146 U/ml upon varying the agitation speed from 100 rpm to 300 rpm. However, the best fungal growth was achieved at 100 rpm. The results suggested that the enzyme was non growth associated (Purwanto *et al.*, 2009). The agitation speed that was higher than 200 rpm shown that have a low enzyme production and this condition could be due to the fungal cell disturbances that caused by shear stress or shear forces. Besides, lower the agitation speed also resulted in low growth, which resulted in low tannase production. This could be due to low amount of dissolved oxygen in cultivation medium (Purwanto *et al*., 2009). According to Wang *et al.* (2005), in much fungal fermentation, a higher agitation rate was necessary to provide adequate mixing and mass transfer, especially when the fungal cells grew in a freely dispersed form which resulted in a non-Newtonian broth and higher apparent viscosity. The vigorous agitation seems to affect the catalytic activity of the enzymes (Darah *et al.*, 1997). However, the agitation speed should be limited to a range that could avoid exerting high shear stress on fungal mycelia.

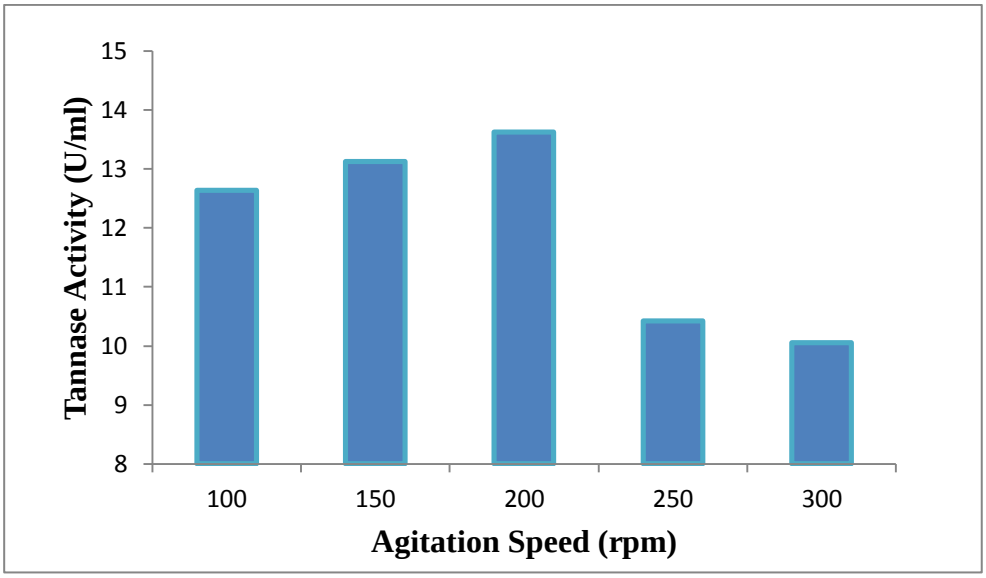

Figure 4.3: Effect of agitation speed on tannase activity

4.3 OPTIMIZATION USING RSM

According to Saravanakumar *et al.* (2010), studying one-factor-at-a-time will take a long period of time and quite costly, when a large number of variables are evaluated. In order to fully exploit the enzymatic reaction it is decided to study the experimental regime from a statistical stand-point, and designs a protocol using response surface methodology (RSM). This technology is useful model for studying factors that affect the response by varying them simultaneously and it can also be used to study the relationship between one or more factors (independent variables) and responses (dependent variables) (Hatijah, 2010).

Three experimental factors, pH, substrate concentration and agitation speed were chosen for optimization of tannase activity by the *Aspergillus niger*. Response surface methodology using three factors and five level central composite designs were used to optimize the response of variables. Each factor in the design was studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) (Table 4.1). All variables were taken at central coded value and considered as zero. The minimum and maximum ranges of variables were investigated and the full experimental plans with respect to their value in actual and coded form were listed in Table 4.1. The tannase activity was studied as response. This

criterion was used in all experimental designs and analyzed with the aid of Design Expert ver. 6.0.6 statistical software (Stat-Ease Inc, Minneapolis, MN). The tannase activity was analyzed using of variance (ANOVA). The optimum levels of variables were obtained by graphical and numerical designs using Design expert program.

Table 4.1: Experimental range and level coded of process variables

Independent variables	**Units**	**Symbol**	**Ranges and levels**				
			-∞	-1	0	+1	+∞
pH	-	X_1	2.65	3.50	4.75	6.00	6.85
Substrate concentration	g/l	X_2	0.73	2.00	6.00	10.00	12.73
Agitation speed	rpm	X_3	31.82	100.00	200.00	300.00	368.18

Table 4.2 shows the design matrix of the variables and the experimental results for tannase activity of *Aspergillus niger*. The design independent variables and their range were classified as in Table 4.1.

Table 4.2: Central composite design (CCD) of factors with tannase activity as response

Standard	Run	Block	pH	Substrate Concentration (% w/v)	Agitation Speed (rpm)	Predicted	Tannase Activity (U/ml)
9	1	Block 1	2.65	6.00	200.00	8.54	7.00699
16	2	Block 1	4.75	6.00	200.00	14.46	15.3131
18	3	Block 1	4.75	6.00	200.00	14.46	14.9937
20	4	Block 1	4.75	6.00	200.00	14.46	11.9867
15	5	Block 1	4.75	6.00	200.00	14.46	14.2489
3	6	Block 1	3.50	10.00	100.00	8.60	9.98869
11	7	Block 1	4.75	0.73	200.00	8.50	7.88980
4	8	Block 1	6.00	10.00	100.00	12.58	11.9055
6	9	Block 1	6.00	2.00	300.00	8.76	7.75241
7	10	Block 1	3.50	10.00	300.00	8.31	8.07188
2	11	Block 1	6.00	2.00	100.00	9.73	10.3467
12	12	Block 1	4.75	12.73	200.00	12.49	12.5607
17	13	Block 1	4.75	6.00	200.00	14.46	15.2067
1	14	Block 1	3.50	2.00	100.00	7.07	7.73901
14	15	Block 1	4.75	6.00	368.18	6.22	6.68909

Table 4.2:-Continued

Standard	Run	Block	pH	Substrate Concentration (% w/v)	Agitation Speed (rpm)	Predicted	Tannase Activity (U/ml)
5	16	Block 1	3.50	2.00	300.00	6.41	7.46715
8	17	Block 1	6.00	10.00	300.00	11.98	11.6925
13	18	Block 1	4.75	6.00	31.82	7.27	6.26164
10	19	Block 1	6.85	6.00	200.00	13.86	14.8425
19	20	Block 1	4.75	6.00	200.00	14.46	15.1326

Using multiple regression analysis on the experimental data, the following second-order polynomial equations (4.1) was found to shown the tannase activity as (Y).

$$Y = 14.46 + 1.58A + 1.19B - 0.31C - 1.16A^2 - 1.40B^2 - 2.73C^2 + 0.33AB - 0.077AC + 0.092BC \quad (4.1)$$

where Y (U/ml) was the response factors for tannase activity. A, B, and C were values of independent factors for pH, substrate concentration (% w/v) and agitation speed (rpm). In order to verify the validity of the models, it is necessary to conduct an analysis of variance (ANOVA) as shown in Table 4.3.

Table 4.3 recorded that the regression for tannase activity were significant at 3.02 and those lacked of fit were not significant at p is 0.3507 and the values are greater than 0.1000 that indicate the model terms are not significant. The fit of the models were checked by the correlation coefficient, R^2. The R^2 value provided a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value always lied between 0 and 1. The closer R^2 value to 1.00, the stronger the model was and the better it predicted the response. In this case, the R^2 value for tannase activity is 0.9111. These values showed that 7.52 percent of the total variable was not explained by the model. The '$Pred\ R^2$' of 0.5502 for tannase activity was reasonable agreement with the '$Adj\ R^2$' of 0.8311. This indicated a good agreement between the experimental and predicted values for tannase activity. The adjusted R^2corrected the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeable smaller than R^2. This should be a caution signal as too many terms were present in the model (Haaland, 1989).The plot of predicted versus experimental tannase activity are shown in Figure 4.4 with R^2 =0.9111, thus indicating an excellent adequacy of the proposed model.

Table 4.3: ANOVA for response surface quadratic model

Source of variations	Sums of squares	Degrees of freedom	Mean square	*F*-value	Significance (*P* value)
Tannase Activity (Y)					
Regression	190.67	9	21.19	11.39	0.0004
Residual	18.61	10	1.86		
Pure Error	8.18	5	1.64		
Lack of Fit	10.43	5	2.09	1.27	0.3984
Total	209.28	19			

Tannase Activity: R^2 0.9111; adjusted R^2 0.8311; predicted R^2 0. 5502

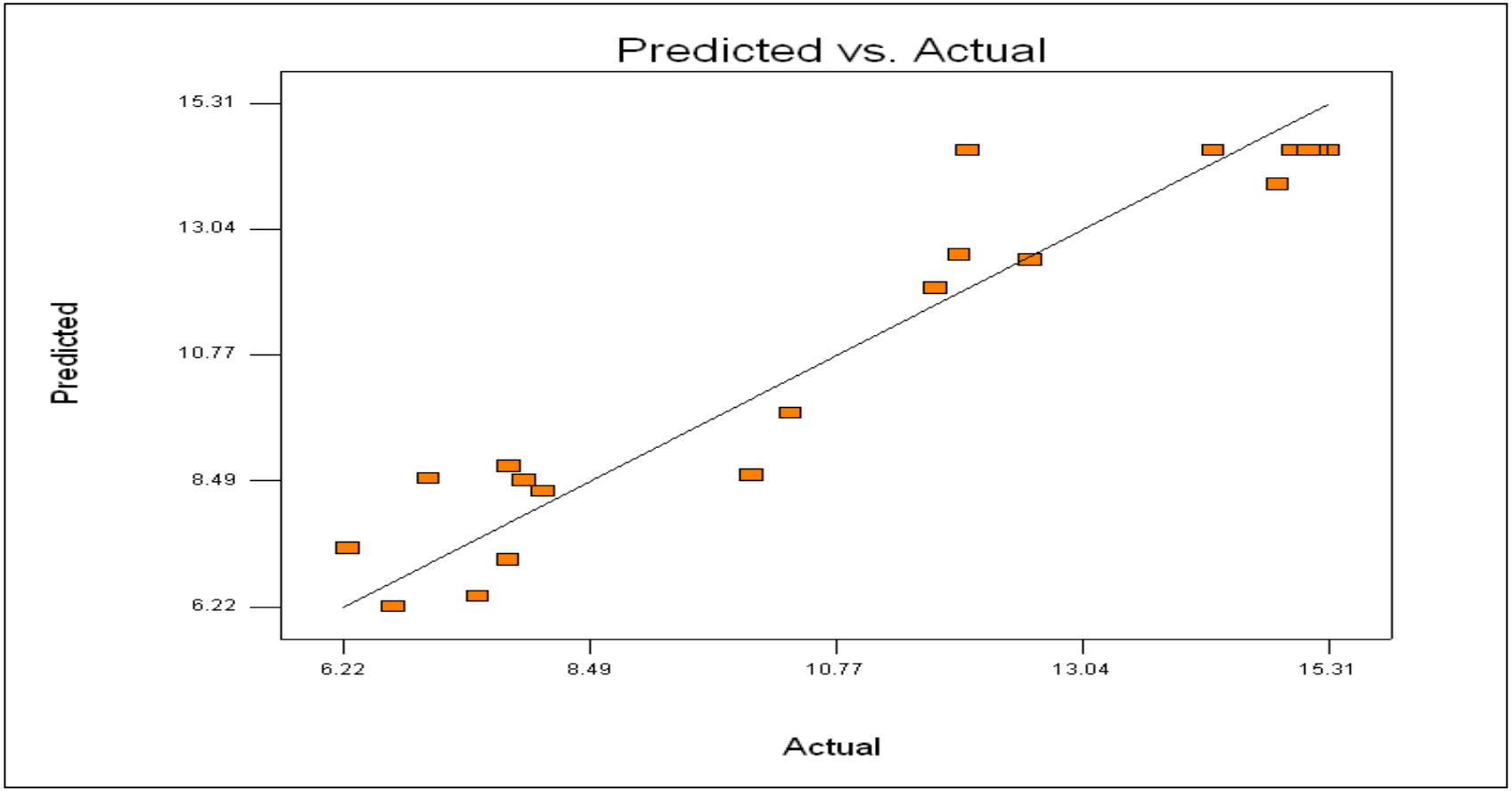

Figure 4.4: Plot of predicted versus experimental data for tannase activity

4.3.1 INTERACTION OF PARAMETERS

The three-dimensional response surface curves were plotted to study the interaction among different parameters, and to study the optimum condition for tannase activity.

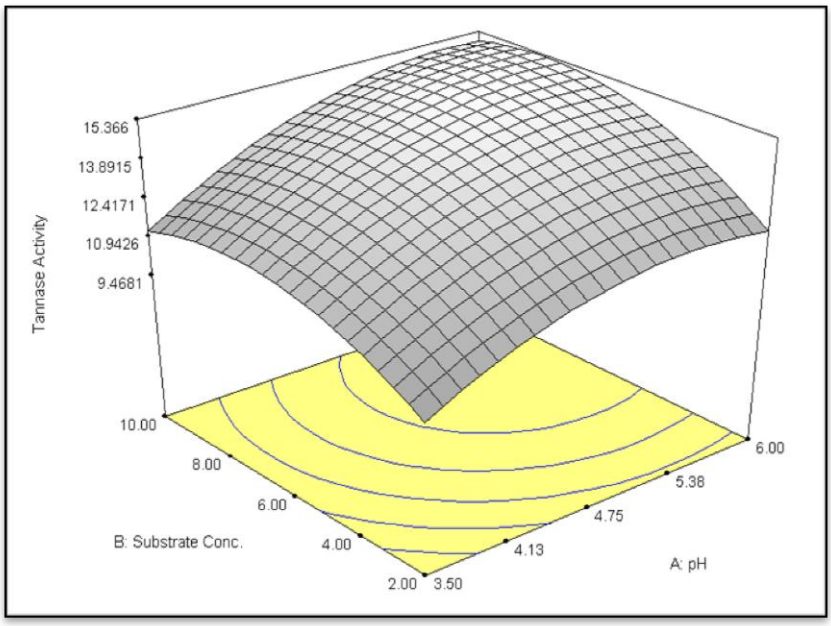

Figure 4.5: Interaction of pH and substrate concentrations towards tannase activity

Figure 4.5 shows the response of tannase activity with respects to pH and substrate concentration. The highest enzyme activity obtain from Figure 4.5 was in pH (4.75) and substrate concentration (6.0w/v %) induced to the tannase activity to 14.9055 U/ml. However, the enzyme activity tends to reduce as the pH decreased to 3.5 and at low glucose concentration was lower (2.0 w/v %.). This was due to the change in pH that affects the protein structure and reduces the enzyme activity due to enzyme inactivation or its instability (Mahapatra *et al.*, 2005). The research done by Lokeswari (2010) shows that when the substrate concentration at 0.5w/v %, the activity of the tannase was high compare to 0.01 w/v % and 1.5 w/v %. Beverini *et al.* (1990) mentioned that lower concentration of glucose is not repressive for enzyme production in *Aspergillus japonicas*. However, a high substrate concentration showed a great affect in inhibiting tannase activities.

From the previous research, the highest tannase activity was obtained after the complete consumption of glucose. According to Lekha and Lonsane (1997), tannase enzyme in an audible enzyme in fungus and produced only in the presence of tannic acid. The enzyme degrades the tannic acid into gallic acid and glucose, which is ultimately utilized by the organism for growth. The *Aspergillus niger* also able to grow tannic acid with initial pH of 3.0 to 7.0. The maximum tannase activity was observed at pH 4.75. Earlier Lekha and Lonsane (1997) also mentioned that tannase is an acidic protein with optimum pH around 5.5. This is in agreement with the result of other researcher (Lokeswari, 2010) who used *Aspergillus oryzae* as their tested strain.

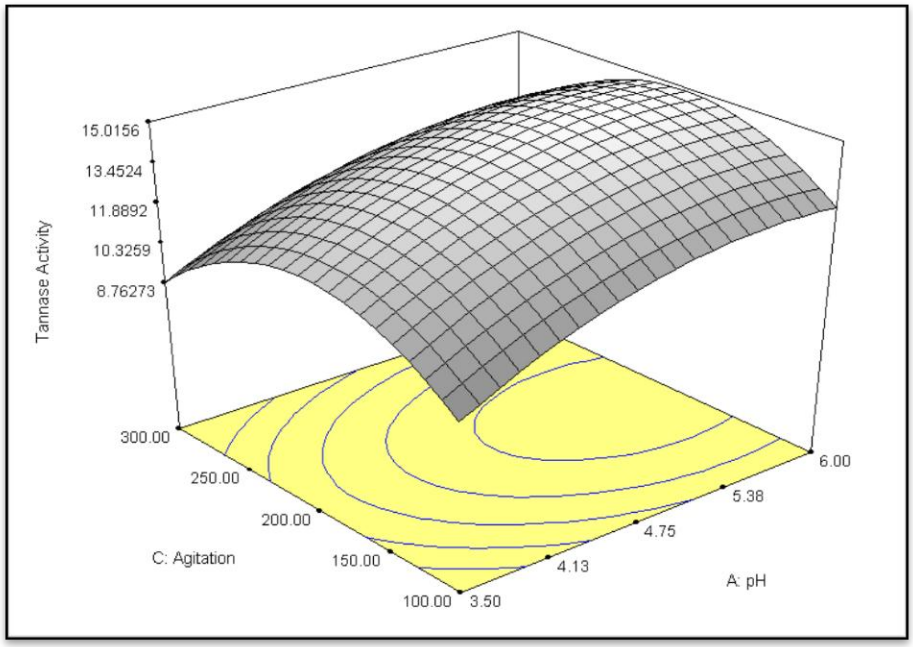

Figure 4.6: Interaction of pH and agitation speed towards tannase activity.

Figure 4.6 shows the effects of agitation speed and pH towards tannase activity. The enzyme activity at 200 rpm has the highest enzyme activity with the pH of medium was 4.75. The agitation speeds on the submerged fermentation give aeration to the solution (Purwanto *et al*, 2009). The activity of the enzyme dropped rapidly when the agitation

speed and pH increased. Lee and Chen (1997) mentioned that enzyme seemed to be affected by higher agitation speed as it may increase shearing effects into cells. As mentioned previously, the higher agitation speed increased higher amount of dissolve oxygen and dispersion of macromolecules in the medium. It will lead to the more growth and activity enzyme. However, the agitation speed has their limit that can affect their shearing effect on the cells and enzyme activity. It also will inhibit the cell growth and enzyme stability. In fact, similar result also can be observed in this study. (Purwanto *et al*., 2009; Hatijah, 2010)

In Figure 4.6, hemisphere shape of three dimensional plots inferred that interaction between pH and agitations were evident with significant linear correlations. Higher tannase activity was obtained at pH 4.0 to 6.0. The Figure 4.6 shows at acidic pH and slow agitation speed, and at pH 6 and 300 rpm of agitation speed it only produces a little of enzyme activity and at pH 6 and 300 rpm of agitation speed. Belmares *et al*. (2004) mentioned pH 3.5 to 6.0 was a stable pH for the determined their enzyme activity. Usually, the effect of pH on microbial growth may attribute to the hydrogen (H^+) concentration. H^+ can be considered as substrate under the pH range 6.0 to 7.0 but acts as inhibitor under the acidic and alkaline pH (Mahapatra *et al*., 2005). Previous research mentioned that the enzyme has almost no enzyme activity below pH 3.0 or above pH 10.0 (Hatijah, 2010).

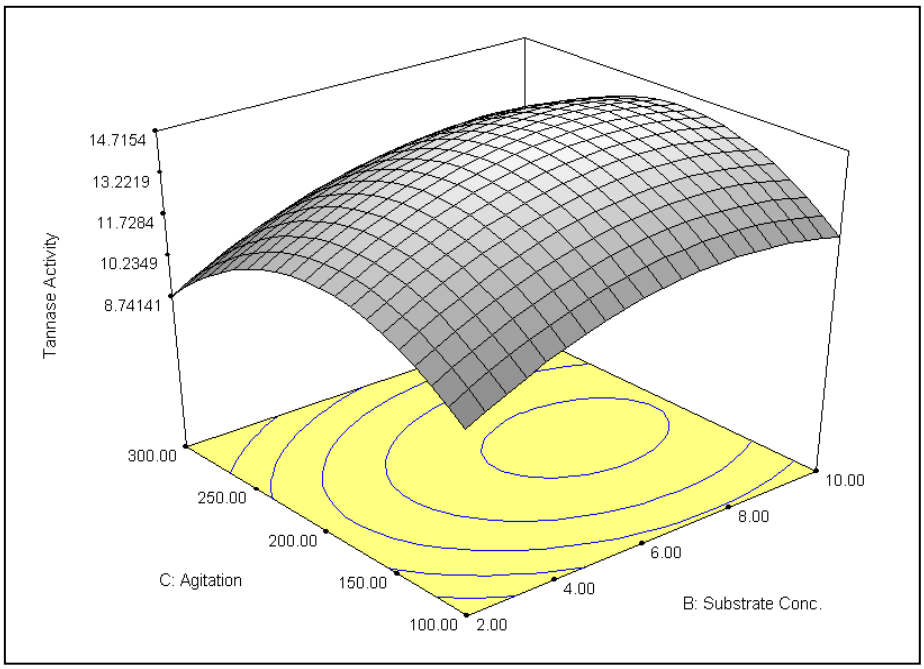

Figure 4.7: Interaction between substrate concentration and agitation speed towards tannase activity.

In Figure 4.7, the graph shows the interaction between substrate concentration and agitation towards tannase activity. The maximum tannase activity can be obtained at 6 w/v % of glucose concentration and 200 rpm of speed agitation at 14.7054 U/ml.The enzyme activity suddenly decreased when the agitation speed and substrate concentration increased. Purwanto *et al.*, (2009) has mentioned that the agitation speed more than 200 rpm will resulted in low enzyme production and this condition could be due to fungal cell disturbances caused by shear stress or shear force. The production of enzyme also decreased when the substrate concentrations increased. The addition of carbon sources such as glucose to the culture medium at initial concentration will improved tannase production by *Aspergillus niger*. So, the agitation speed will cause the aeration to medium and according to *Belmares et al.*, (2004), tannase production in submerged culture by *Aspergillus niger* is improved at high aeration rates. From Figure 4.7, when the substrate concentration and the agitation speed increased, the tannase activity will decrease.

(Purwanto *et al.*, 2009) stated that the increased in agitation speed will give a higher shear stress, causing the fungal to grow in pellets of smaller size and dense.

4.3.2 VALIDATION OF THE MODEL

In order to validate the adequacy of the model equations (4.1), a total of three verifications experiments for tannase activity response were carried out under various fermentation conditions as shown in Table 4.4.The verification of the results was accomplished by carrying out the experiments under optimal conditions of pH 4.75, 6v/w % of glucose concentration and 200 rpm. The three replicate experiments yield 14.4065 U/ml. The agreement reach between the predicted and experimental results verifies the validity of the model and existence of an optimal point with error from 0.009 to 0.038 (Table 4.4).

Table 4.4: Validation of the data and models constructed for tannase activity

Run				**Tannase Activity**		
	pH	**Substrate Concentration (w/v %)**	**Agitation (rpm)**	**Exp**	**Predicted**	**Error (ε)**
1	4.75	6.00	200	15.032	14.46	0.038
2	4.75	6.00	200	14.947	14.46	0.033
3	4.75	6.00	200	13.239	14.46	0.009

4.4 CELL DRY WEIGHT

After two days of fermentation, the weight was determined in order to observe the optimum growth profile of *Aspergillus niger* in the fermentation medium. Figure 4.8 show the graph of the cell dry weight versus the time of fermentation. The production of tannase was also observed by plot it in the Figure 4.8 as secondary y-axis.

From Figure 4.8, the samples were taken every 8 hours starting from 0 hours to 48 hours. Then, the biomass was obtained by dried at 80 ^{o}C and the cell dry weight was

determined. The growth of *Aspergillus niger* in this fermentation is proportional with the time. In the first 16 hours, the growth of biomass seems to be slow. This phase was known as the lag phase. At this phase, the fungi still did not grow but in the process of adaptation of adaptation with the surrounding. After 16 hours, the mass of biomass starting to growth. This is called phase of exponential or as known as log phase. At log phase, the fungi started to used up the nutrient provided from the fermentation medium and start to grow until it reach their maximum growth at 40 hours. After 40 hours, the fungi faced the stationary phase where the weight of the biomass was maintained and decrease after it started to death phase.

In Figure 4.8 also shows the supernatant was analyzed for tannase production represented by absorbance reading. The enzyme was generated along exponential phase of *Aspergillus niger*. At 40 hours, the absorbance was the highest with 0.109 nm. This is proved that the tannase enzyme is the primary metabolite as it was produced during the active growth of the fungi and the enzyme production was growth along. It is important to study the maximum growth for the fungi to determine the maximum tannase production. So, the maximum growth of *Aspergillus niger* and maximum tannase production was at 40 hours.

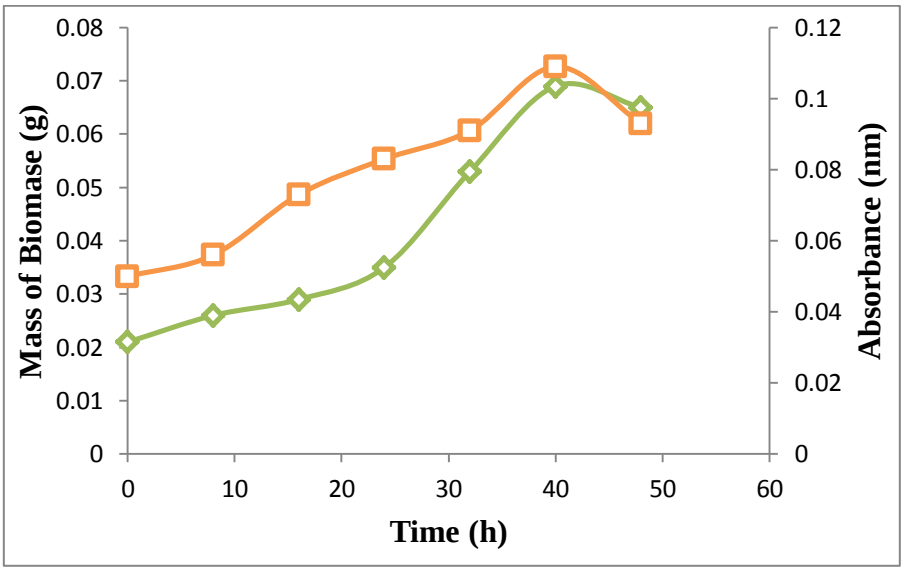

Figure 4.8: Growth profile of *Aspergillus niger* and tannase enzyme production

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

As a conclusion, the production study of extracellular tannase enzyme in shake flask fermentation using glucose as substrate for *Aspergillus niger* was successfully achieved. The study of extracellular tannase production were done by three parameters, pH, glucose concentration and agitation speed to obtain the optimization. For the study of effects of pH for extracellular tannase production in shake flask fermentation showed that the most suitable pH was at pH 5 where it was produced the maximum tannase enzyme at 14.9521 U/ml. At the pH 5, the tannase activity is stable and is it to be an acidic protein. For the study of effects of substrate concentration controlling to the extracellular tannase production in shake flask fermentation showed that maximum tannase enzyme was produced at 6 v/w % and the value of tannase activity was at (13.3234 U/ml). Too high and too low level of glucose concentration is not suitable for enzyme production. For the study of effects of pH to the extracellular tannase production in shake flask fermentation showed the suitable speed of agitation was at 200 rpm where it produced the maximum at 13.2146 U/ml of tannase activity. The agitation speed that exceed 200 rpm produced a low enzyme production that caused by shear stress. The optimization of extracellular tannase activity was produced at pH 4.75, 6 v/w % glucose concentration and 200 rpm by using response surface methodology (RSM) where it showed the maximum value at 15.3131 U/ml.

5.2 RECOMMENDATION

For the future research, the usage of glucose can somehow be substituted to other cost effective carbon source for example waste from agricultural industry to produce higher amount of extracellular tannase. The other microorganism also can be used to obtain the higher amount of extracellular tannase.

On industrial point of view, there are new technology can be applied to improve the production of tannase enzyme. This study must progress by using bioreactor to obtain the higher productivity compare to the shake flask study. The advantages by using bioreactor are it can regulate the agitation and aeration process. The small production of tannase activity is because of limited aeration in the fermentation medium that can cause the death of the cells. Besides that, bioreactor makes the sterilization and sampling processes become easy within.

For further the study on extracellular tannase enzyme, it is recommended to do research about toxicology test of tannase enzyme for industry applications. *Aspergillus niger*, is known production organisms used in the food industries. Toxins of fungal origin can evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a ingestion, or skin contact. Nowadays, the safety of any food-grade product is carefully evaluated before its commercialization. A safety assurance measure consists of the selection of raw materials to manufacture a series of toxicological tests to ensure the safety of food-grade enzyme. So, the studied about toxicology test of the enzyme is very important for application in food industry for the safety of their customers.

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APPENDIX A

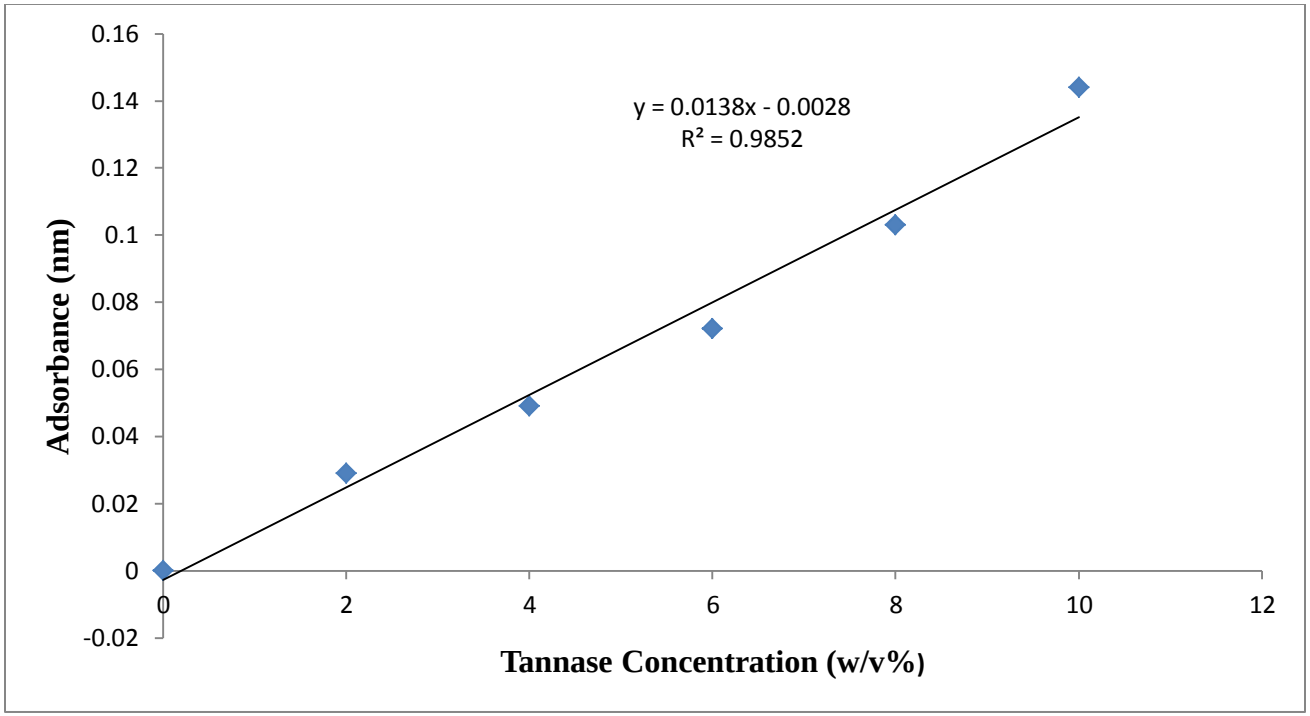

Figure A: Graph of standard curve

APPENDIX B

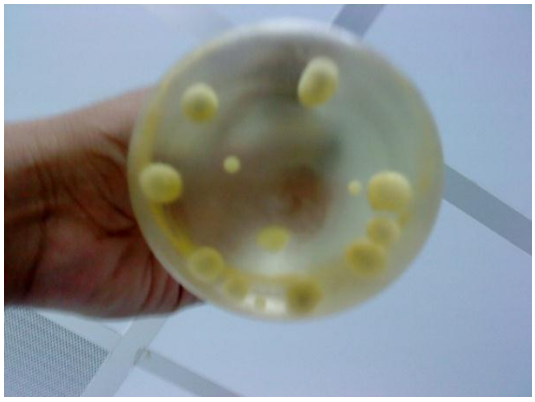

Figure B1: The effect of agitation speed at 36.8 rpm

Figure B2: The effect of agitation speed at 100 rpm

Figure B3: The effect of agitation speed at 200rpm

Figure B4: The effect of agitation speed at 300 rpm

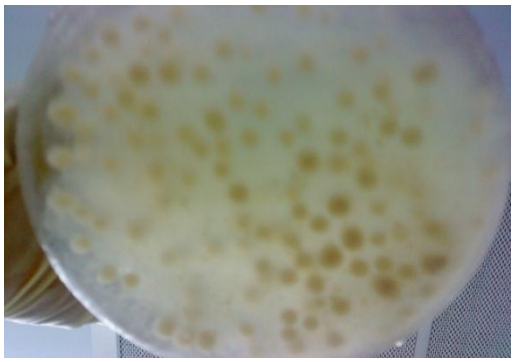

Figure B5: The effect of agitation speed at 368.8 rpm

APPENDIX C

Figure C: The growth of the *Aspergillus niger*