PURIFICATION OF FRUCTOSYLTRANSFERASE (FTase) FROM *ASPERGILLUS NIGER* TO ENHANCE PRODUCTION OF FRUCTOOLIGOSACCHARIDES (FOS) AS A FOOD ADDITIVE

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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 (Biotechnology)

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

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In The Name of Allah, Most Gracious, Most Merciful

Love special dedicated to…

Special inspiring and special encouraging of my lovely parent: Ramli Bin Awang and Razina Binti Muhd Junus;

My siblings (long, alang, akak, kakcik, ngah, kakyah, adik),

and

also my truly best friends,

Those who has influenced my life on the right course

Thank you so much

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ABSTRACT

Nowadays, worldwide consumers are becoming increasingly aware of the relationship between food or food constituents and health. In response to an increasing demand from the consumer, fructooligosaccharides (FOS) have emerged primarily because of its functional properties rather than sweeteners. The enzyme source synthesis can be divided into two classes which are from plant and microorganism. Microorganism producing FOS from FTase had captured much attention from industrial level due to mass production and controlled environment rather than FOS produced from plant. Many researchers produced crude FTase enzyme to produce FOS. In view of that, purification of crude enzyme from microfungi is studied in order to obtain high yield of FOS. This study is carried out using molasses as a substrate in the fermentation process to produce crude enzyme from microorganism. The series of purification step will be done in order to purify the enzyme and to determine the characteristic of purified enzyme. By using an extracellular enzyme which is fructosyltransferase (FTase) from selected micro fungi, the FOS can be produced commercially from sucrose. The enzyme was able to transfer the fructosyl group from sucrose as donor producing corresponding series of FOS: 1-kestose, nystose and fructosylnystose. Although these proteins differ in their subunit structure, molecular weight, chemical susceptibility and substrate specificity, they all display both hydrolytic and transfer activities which limit the FOS production to the use of high sucrose concentration. The optimum pH and temperature for activity of FTase is between 5 to 6.5 and 50 °C to 60 °C with yield of 125 U/mL when crude FTase is used and 136.7 U/mL when purified FTase is used. For the purification studies of FTase, different micro fungi will produce different characteristic of enzyme. The enzyme was able to transfer fructosyl groups from sucrose and then catalyze the formation of short chain FOS.

ABSTRAK

Pada masa kini, para pengguna di seluruh dunia semakin sedar tentang hubungan antara makanan atau kandungan makanan dengan kesihatan. Sebagai respon kepada permintaan yang meningkat daripada pengguna, penggunaan fruktooligosakarida (FOS) telah meningkat terutamanya kerana mempunyai sifat-sifat yang berfungsi dan bukan hanya sekadar pemanis. Sumber sintesis enzim boleh dibahagikan kepada dua kelas yang terdiri daripada tumbuhan dan mikroorganisma. Mikroorganisma yang menghasilkan FOS daripada FTase telah mendapat perhatian daripada sektor perindustrian disebabkan oleh penghasilan yang berskala dan persekitaran yang terkawal berbanding dengan FOS yang dihasilkan daripada tumbuhan. Ramai pengkaji menghasilkan enzim FTase mentah untuk menghasilkan FOS. Sehubungan dengan itu, penulenan enzim mentah daripada kulat dikaji untuk mendapatkan hasil FOS yang tinggi. Kajian ini dijalankan menggunakan gula pekat yang terhasil daripada proses penapisan gula sebagai substrat dalam proses penapaian untuk menghasilkan enzim mentah daripada mikroorganisma. Beberapa siri penulenan dilakukan untuk menulenkan enzim dan untuk menentukan ciri-ciri enzim yang tulen. Dengan menggunakan enzim luar sel iaitu FTase daripada kulat yang dipilih, FOS boleh dihasilkan secara komersial dengan menggunakan sukrosa. Enzim mampu memindahkan kumpulan fructosyl dari sukrosa sebagai penderma menghasilkan siri FOS: 1-kestose, nystose dan fructosylnystose. Walaupun protein ini berbeza dalam struktur subunit mereka iaitu berat molekul, pendedahan terhadap bahan kimia dan substrat yang spesifik, mereka semua menunjukkan kedua-dua aktiviti hidrolisis dan pemindahan yang menghadkan penghasilan FOS kepada kepekatan sukrosa yang tinggi. pH dan suhu yang optimum untuk aktiviti enzim FTase antara 5 ke 6 dan 50 °C ke 60 °C dengan aktiviti sebanyak 125 U/mL bagi enzim mentah dan 136.7 U/mL apabila menggunakan enzim yang tulen. Bagi kajian penulenan FTase, kulat yang berbeza akan menghasilkan enzim dengan ciri-ciri yang berbeza. Enzim mampu untuk memindahkan kumpulan fructosyl dari sukrosa dan kemudian menjadi pemangkin kepada pembentukan rantaian pendek FOS.

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GF_2	1-kestose
GF_3	nystose
GF_4	1-β-fructofuranosylnystose
2^n	Factorial run
2n	Axial run
n_c	Center runs

LIST OF ABBREVIATION

ANOVA	Analysis of variance
CCD	Central composite design
DEAE	Diethylaminoethyl
DNS	Dinitrosalicyclic acid
FFase	Fructofuranosidase
FFT	Fructan-1-fructosyltransferase
FOS	Fructooligosaccharides
FOSHU	Food of Specified Health Used
FTase	Fructosyltransferase
OD	Optical density
PDA	Potato dextrose agar
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SST	Sucrose 1-fructosyltransferase

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Fructooligosaccharides (FOS) are oligosaccharides of chain of fructose containing a single glucose molecule (L'Hocine et al., 2000). Fructooligosaccharides (FOS) is mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1-β-fructofuranosylnystose (GF_4). They are produced by the action of enzyme fructosyltransferase (FTase) from plants and microorganisms. FOS has functional properties such as low calorific values, non-cariogenic properties, decrease level of phospholipids, triglycerides and cholesterol, help gut absorption of calcium and magnesium. These properties are useful for diabetic's products and used as prebiotics (Sangeetha et al., 2005; Sanchez et al., 2008). Fructooligosaccharides are industrially produced from sucrose by microbial enzyme with transfructosylating activity mainly found in fungi such as *Aureobasidium* sp., *Aspergillus* sp., *Arthrobacter* sp. and *Fusarium* sp. (Park et al., 2001). The enzyme source synthesis can be divided into two classes which are from plants and microorganisms. The enzyme source from plant such as sugar beet leaves, lettuce (Sangeeetha et al., 2005) and asparagus roots, onion bulbs (L'Hocine et al., 2000), FTase is produced intra- and extracellularly by several fungi. To optimize the production of FOS, the cost must be reduced and in this study, the evaluation with strain *Aspergillus niger* was carried out on molasses as a source of sucrose because pure sucrose is expensive rather than molasses (Shin et al., 2003). In view of that, purification of crude enzyme from microfungi using molasses as a substrate is studied in order to obtain high yield of FOS. The series of purification step will be done in order to purify the enzyme and to determine the characteristic of purified enzyme.

1.2 PROBLEM STATEMENT

Normally, pure sucrose is expensive and the cost to produce FTase by using sucrose is high. There are some by-product like cereal bran, corn-products, sugarcane molasses and by-products of coffee and tea processing industries were used as a substrate to produce FTase from microorganism (Sangeetha et al., 2003). This by-product can lower the cost of production of FTase. Before FOS can be produced, the enzyme FTase must be undergone a series of purification. The purification of FTase must be done in order to get high yield of FOS. Many researchers have reported the purification and characterization of FTase from various sources and FTase has been found to differ in their molecular weight and properties from one source to another (Lateef et al., 2006, Sangeetha et al., 2003).

FTase exhibits hydrolytic activity which can dominate the process (Delphine et al., 2007). This fact will lead to lower production yields and to a contamination of the final product with glucose and fructose (Delphine et al., 2007). Purification of FTase is important for batch production of FOS. By doing so, the nature of its hydrolytic activity can be studied, improve the understanding its mode of operation and be able to classified which type of enzyme should it belong to (L'Hocine et al., 2000).

1.3 RESEARCH OBJECTIVES

The main objective of this research is to purify FTase enzyme from microfungi *Aspergillus niger* isolated from bread in order to enhance the production of FOS as a food additive.

The measurable objectives are to determine:

1. The effect of temperature towards the enzyme activity.
2. The effect of substrate concentration towards the enzyme activity.
3. The effect of pH towards the enzyme activity.

1.4 SCOPE OF STUDY

Purification of crude enzyme from microfungi is studied in order to obtain high yield of FOS. This study is carried out using molasses as a substrate in the fermentation process to produce crude enzyme from microorganism. The series of purification step consist of 5 steps will be done in order to purify the enzyme and to determine the characteristic of purified enzyme. The microorganism is maintained on agar slants at 4°C. The inoculum is developed by transferring the mycelia from a 3-days old slant into the inoculum medium consist of 1% sucrose, 0.2% yeast extract at pH 5.50. The flask was incubated for 24 h at 30°C on a rotary shaker.

After 24 h, the inoculum was transferred into 100mL of fermentation medium fermentation medium consists of 17.5% w/v sucrose, 1% w/v peptone, 0.5% w/v yeast extract, 0.1% $MgSO_4.7H_2O$ and KH_2PO_4). The inoculum will undergo fermentation for 48 hours at 30 °C in incubator shaker at 250 rpm. Biomass was harvested by filtering culture broth using filter paper. The cell free fluid was taken as a source of crude extracellular enzyme.

The crude enzyme is purified and characterized based on the enzyme stability towards temperature, substrate concentration and cultivation time. The parameters are optimized by using Response Surface Methodology (RSM). Then, the activities of crude and purified enzyme are compared for FTase activity of both enzymes. The FTAse activity is analyzed by using correlation of glucose standard curve from UV-Vis Spectrophotometer.

1.5 SIGNIFICANCE OF STUDY

In Malaysia, the FTase enzyme used to be imported from other country such as Japan, India and United States (Sangeetha et al., 2005). Normally, the cost for production of FOS from commercialized enzyme is high, so by producing and purifying the enzyme, we can reduce the cost. The significance in this study is the raw material or substrate used

is molasses which is a by-product and considered as waste from sugar refinery process. Price of sucrose is quite expensive which is RM 2.30 per kilo (Norliza, A.R. 2011. Rasionalisi subsidi: Harga gula naik 20 sen esok. *Utusan Malaysia*. 9 May.) while molasses is only RM 1.50 per kilo (Husin. 2011. Molasses. http://gulamolasses.blogspot.com). In order to extend the usage of FOS, it is necessary to minimize the production cost. Molasses from sugar beet processing industry are cheap and readily available source of sucrose. By using molasses, we could reduce the cost of FTase production and concurrently we can help the sugar industry to improve their economy and manage their waste properly. By using concept of waste into wealth, molasses which is waste from sugar industry was being used to make it valuable.

Normally, crude enzyme will produce lower yield of FOS than purified enzyme. In order to fulfill high customer demand on the functional food such as FOS, the production of FOS must be increased. In order to produce high yield of FOS, the enzyme need to be purified. By doing this, the activity of enzyme will be increased and so do the production of FOS.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The production of fructooligosaccharides (FOS) has been received particular attention because of their beneficial for health and their functional properties effects. Those effects such as activation of the human immune system, resistance to infection, enhanced mineral absorption in the gastrointestinal tract, lowering of serum cholesterol and preventing carcinogenic tumors (Qiang et al., 2009). Fructooligosaccharide (FOS) has broad application in food industries due to their prebiotic properties and they are 0.4 to 0.6 times as sweet as sucrose and have been used as a functional sweetener in pharmaceutical industry (Sanchez et al., 2008). It is because of their prebiotics properties which is defined as 'non-digestible' food that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson and Roberfroid, 1995). Prebiotics also has been used to stimulate the bifidobacteria growth in the human colon (Tomasik, P.J and Tomasik, P. 2003). FOS are useful for diabetic product because of their properties such as low calorific values, non-cariogenic properties that could decrease level of phospholipids, triglycerides and cholesterol and help gut absorption of calcium and magnesium (Sangeetha et al., 2005). In order to optimize the usage of FOS, it is necessary to reduce the production cost which can be achieved by process improvement together with strain development and genetic manipulation (Shin et al., 2003). There are two classes of enzymes that are useful for FOS production at industrial scale which is fructosyltransferase (FTase) and β-fructofuranosidases (FFase) or also called invertase (Ghazi et al., 2006).

2.2 FRUCTOSYLTRANSFERASE (FTase)

FTase is an enzyme that catalyses the transformation of sucrose into fructooligosaccharides (FOS) which are important prebiotic that have a broad application in food and pharmaceutical industries (Sanchez et al., 2008). FTase catalyzes the transfer of fructosyl moieties where a donor or acceptor of these moieties can be sucrose or FOS (Antosova et al., 2002). It is widely used in food industries and pharmaceuticals industries because of their functional properties. In the industrial production of FOS, the cells with the FTase activity are produced by aerobic cultivation of fungi such as *Aspergillus aculeatus* (Nemukula et al., 2008), *Aureobasidium pullulans* (Shin et al., 2003), *Lactobacillus reuteri* (Hijum et al., 2002) and *Aspergillus niger* (L'Hocine et al., 2000). FOS is commonly present in food such as fruits, vegetables, cereals and honey. The increasing interest in prebiotic compound opens possibilities for small scale use of FTase. In recent year, the production of FOS using FTase derived from microorganism has attracted attention of many researchers (Sangeetha et al., 2005). In the industrial production of FOS, the cells with the FTase activity are produced by aerobic cultivation of fungi such as *Aspergillus niger, Aspergillus japonicus* or *Aureobasidium pullulans.* There are many researchers that reporting the optimum pH and temperature for activity of FTase between 5 to 6.5 and 50 °C to 60 °C (Ghazi et al., 2006).

2.2.1 Fructosyltransferase Mechanism

The reaction mechanism of the FTase is depending on the type of source of the enzyme. In plant and some microorganism, a series of enzyme act together whereas a single enzyme works in most of the other microorganism. For example, fructosan metabolism in plant such as Jerusalem artichoke (*H.tuberosus*) is established by two enzymes: sucrose: sucrose 1-fructosyltransferase (SST) and β (2 ⟶ 1) fructan: β (2 ⟶ 1) fructan-1-fructosyltransferase (FFT). In the first mechanism, SST converts sucrose into glucose and an oligofructoside but is unable to promote polymerization above the trisaccharide level (Yun, 1996). Further higher polymers are synthesized by FFT (Yun, 1996). The overall reaction mechanism as follows:

$$GF + GF \rightarrow GF - F + G \quad (2.1)$$

$$GF - Fn + GF - Fm\ GF - F_{n-1} + GF - F_{m+1} \quad (2.2)$$

Where GF is a sucrosyl group and n is the number of extransucrosyl fructose residues.

In the study of microbial organism mechanism, (Yun, 1996) had proposed a mathematical model for the mode of action of FTase derived from *A.pullulans*. The enzyme reaction mechanism is as follows:

$$GF_n + GF_n \rightarrow GF_{n-1} \quad (2.3)$$

Where n= 1-3

According to this mechanism, the enzyme acts on sucrose in a disproportion type reaction where one molecule of sucrose serves as a donor and another acts as an acceptor.

2.2.2 Characteristics of FTase

Several FTase have been extensively purified and characterized (Yun, 1996). The optimum pH and temperature for FTase activity is between 5 to 6.5 and 50 °C to 60 °C respectively (Lateef et al., 2006). The definition of enzyme unit also differs between researchers. Some units are defined as the amount of enzyme responsible for transferring one μmole of fructose per min while others are defined as the amount of enzyme capable of producing one μmole of glucose per min (Yun, 1996). The specificity of microbial FTase depends on the β-D-fructoside residue of sucrose (Yun, 1996). Many FOS-producing microorganisms also simultaneously produce a hydrolytic enzyme that degrades FOS (Yun, 1996). Table 2.1 summarizes the characteristic of purified enzyme from various microbial sources. The enzyme source can be divided into two classes; one is plant and the other consists of bacterial and fungal. Table 2.2 and Table 2.3 summarize the classes of enzyme.

Table 2.1: Characteristic of FTase purified from various microbial sources

Source of FTase	Purification fold	Molecular weight (kDa)	Optimum		Stability		References
			pH	Temperature	pH	Temperature	
Bacillus macerans EG-6	63.5	66	5.0	50 ℃	5.0-7.0	20 ℃ -50 ℃	Park et al., 2001
Arthrobacter oxydans J17-21	95.5	54	6.5	45 ℃	5.0-11.0	20 ℃ -40 ℃	Jang et al., 2003
Microbacterium laevaniformans ATCC 15953	4.6	64	6.0	30 ℃	5.0-7.0	-	Park et al., 2003
Aspergillus niger ATCC 20611	51.6	340	5.0-6.0	50 ℃ -60 ℃	4.5-10.0	Up to 60 ℃	Hirayama et al., 1989
Arthrobacter sp. K-1	405.3	52	6.5-6.8	55 ℃	5.5-10.0	Up to 40 ℃	Fujita et al., 1990
Streptococcus salivarus ATCC 25975	34.5	125.4	6.0-7.0	37 ℃ - 40 ℃	-	-	Song and Jacques., 1999
Microbacterium sp. AL-210	98.8	46	7.0	40 ℃	7.0-8.0	Up to 40 ℃	Cha et al., 2001
Aspergillus niger AS0023	78.5	81-168	5.8	50 ℃	4.5-11.0	30 ℃ -50 ℃	L'Hocine et al., 2000
Aspergillus foetidus	25	-	4.5	60 ℃	4.0-6.0	Up to 40 ℃	Wang and Rakshit, 2000

Source: Sangeetha et al., 2005

Table 2.2: Fructooligosaccharides enzyme from plant

Source	**Authors**
Agave Americana (agave)	Bhatia et al., 1979; Nandra and Bhatia, 1980
Agave vera cruze (agave)	Bhatia et al., 1954,1955; Satyanarayana, 1976
Asparagus officinalis (asparagus root)	Shiomi et al., 1976
Allium cepa (onion bulbs)	Darbyshire et al., 1978; Henry and Darbyshire, 1980
Cichorium intybus (chicory)	Singh and Bhatia, 1971; Chandorkar and Collins, 1972
Crinum longifolium	Bhatia et al.,1959
Sugar-beet leaves	Allen and Bacon, 1956
Helianthus tuberosus (Jerusalem artichoke)	Edelman and Dickerson, 1966; Praznik et al., 1990
Lactuca sativa L. (lettuce)	Chandorkar and Collins, 1972
Lycoris radiate (monocot)	Nagamatsu et al., 1990
Taraxacum officinale (dandelion)	Chandorkar and Collins, 1972

Source: Yun, 1996

Table 2.3: Fructooligosaccharides-producing microorganisms

Microorganisms	**Authors**
Aureobasidium pullulans	Jung et al., 1989; Yun et al., 1990; Smith et al., 1980
Aureobasidium sp.	Hayashi et al., 1989
Arthrobacter sp.	Fujita et al., 1990
Aspergillus japonicas	Duan et al., 1994
Aspergillus niger	Hidaka et al., 1989; Bealing and Bacon, 1953
Aspergillus oryzae	Pazur, 1952; Kida et al., 1988, Bealing and Bacon, 1953
Aspergillus phoenicis	Balken et al., 1991
Aspergillus sydowi	Muramatsu et al., 1988
Claviceps purpurea	Dickerson, 1972; Arcamone et al., 1970
Fusarium oxysporum	Gupta et al., 1982; Maruyama et al., 1979; Patel et al., 1994
Penicillium frequentans	Usami et al., 1991
Penicillium spinulosum	Bealing and Bacon, 1953
Phytophtora parasitica	Hankin and Meintype, 1977
Scopulariopsis brevicaulis	Takeda et al., 1994
Saccharomyces cerevisiae	Straathof et al., 1986

Source: Yun, 1996

2.3 PREBIOTICS

Prebiotics are defined as nondigestible substances of food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or limited number of bacteria in the colon that can improve the host health (Delphine et al., 2007). Prebiotic undergo fermentation by beneficial microorganism in the intestine. The

microorganism as a probiotics while prebiotics as a source of energy for the microorganism. Main targets for the prebiotic are intestinal bacteria which are bifidobacteria and lactobacilli. These two bacteria show several positive effects on human being (Rycroft et al., 2001). The most extensively studied prebiotics is fructooligosaccharides and family of fructose polymers which vary in length and can either be derivatives of simple fructose polymers that attached to the sucrose molecules.

2.4 FRUCTOOLIGOSACCHARIDES

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety which is produced by the action of transfructosylating activity from many plants and microorganisms (Delphine et al., 2007). In response to an increasing demand from the healthier consumer food, alternative sweeteners such as palatinose and various oligosaccharides including isomaltooligosaccharides, soybean oligosaccharides and fructooligosaccharides have emerged since 1980 (Yun, 1996). They are important because of their functional properties rather than sweeteners. Various oligofructosides have been produced by the action of transfructosylating activity from many plants and microorganisms (Shin, 2003). FOS is industrially produced from sucrose by microbial enzyme with transfructosylating activity. Figure 2.1 illustrated the step on how to produce FOS. Most of these enzymes have been found in fungi such as *Aspergillus* sp., *Aureobasidum* sp., *Arthtrobacter* sp. and *Fusarium* sp. (Sanchez et al., 2008 and Sangeetha et al., 2005). Commercial FOS may contain glucose, fructose and sucrose in more than 500g per kg of total FOS dry weight (Sangeetha et al., 2005). The FOS formed contain fructosyl unit bounded at the β-2, 1 position of sucrose and they are mainly composed of 1-kestose, nystose and 1- β-fructofuranosyl nystose (Sanchez et al., 2008; Sangeetha et al., 2005). Functional foods will contain the proper balance of ingredients that will help to improve many aspects of human lives, including the prevention and treatment of illness and disease (Sangeetha et al., 2005). Table 2.4 illustrates the natural sources of FOS and the molecular structure of FOS presented in Figure 2.2.

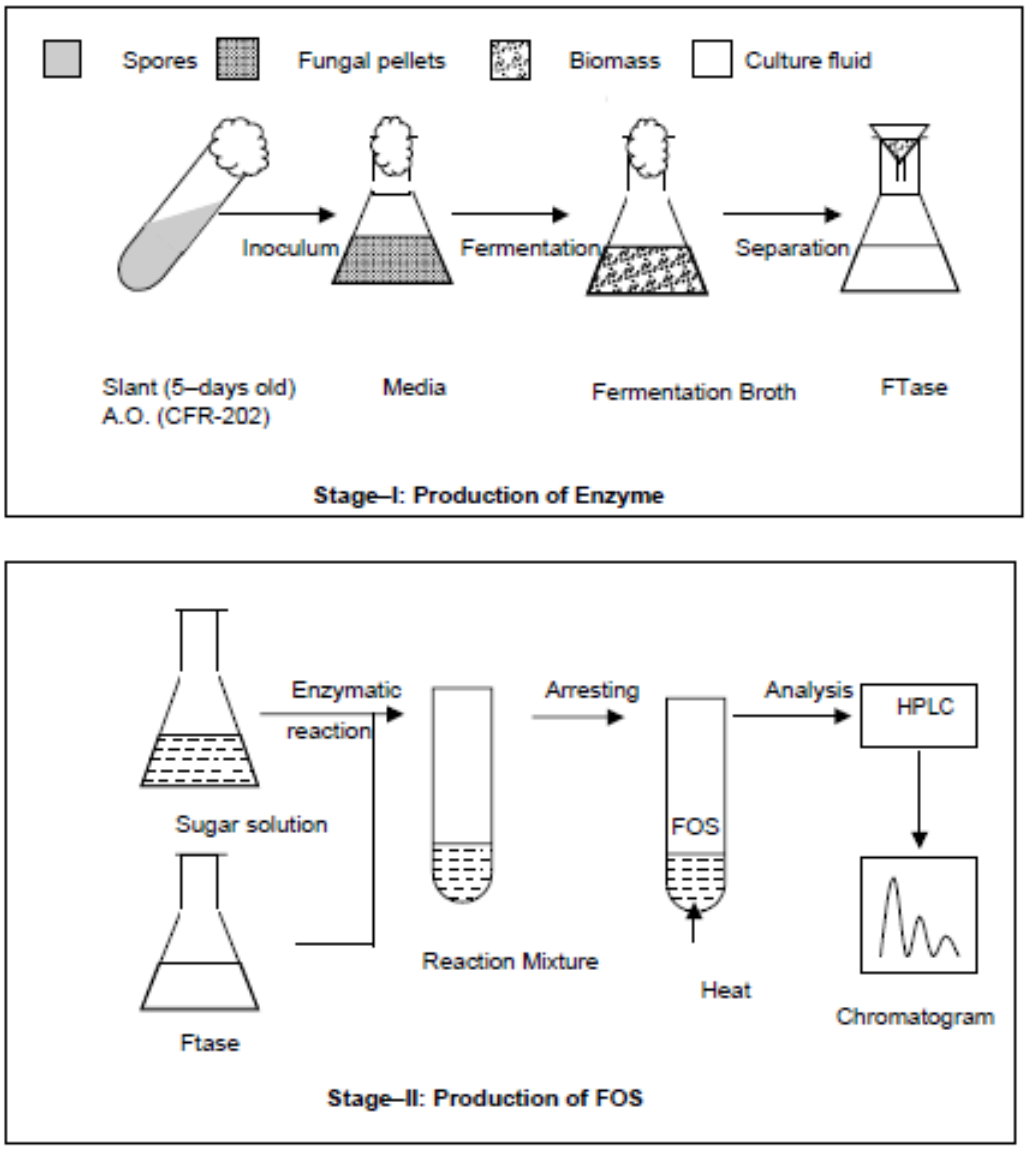


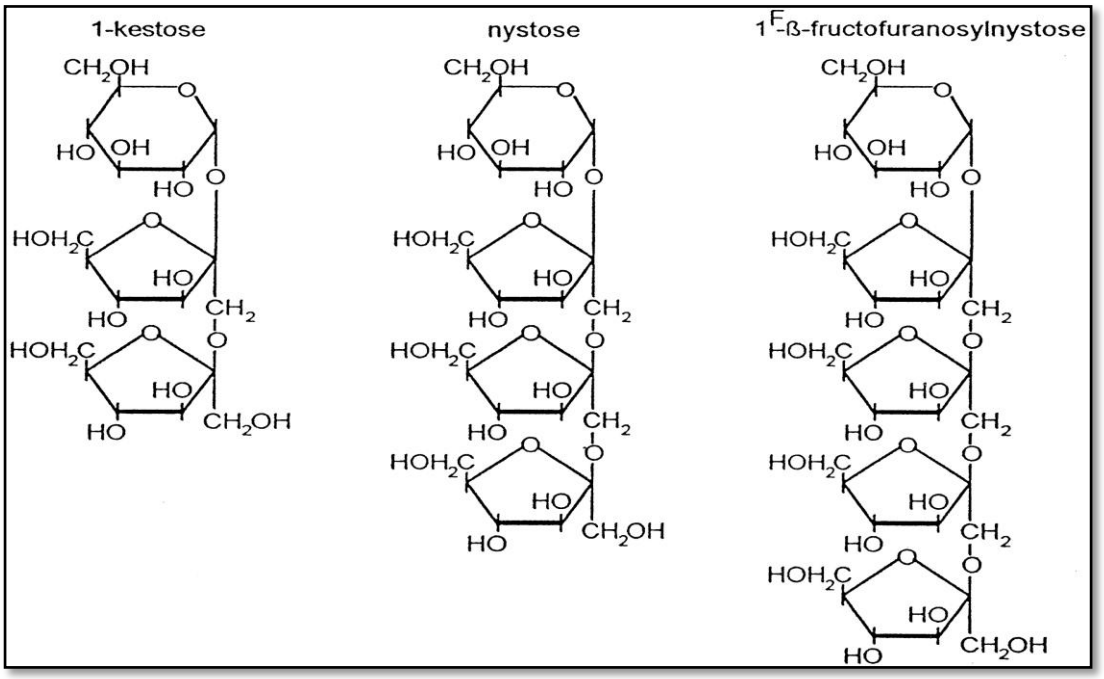
Figure 2.1: Flow chart for producing FOS

Source: Sangeetha et al., 2005

Table 2.4: Concentration of FOS in natural foods

Source	% FOS
Barley	0.15
Tomato	0.15
Onion	0.23
Banana	0.30
Brown sugar	0.30
Rye	0.50
Garlic	0.60
Honey	0.75

Source: Sangeetha et al., 2005



(a) (b) (c)

Figure 2.2: Molecular structure of FOS that consist of 3 main compounds which are a) 1-kestose, b) nystose and c) 1-β-fructofuranosylnystose

Source: Yun., 1996

2.5 MOLASSES

Molasses is a mixture of sucrose and reducing sugars such as glucose and fructose which contain many unidentified components with particulate material. It is a thick, brown to deep black in color, honey-like substance made from leftover residue of the sugar making process. In agricultural industry, molasses is the concentrated unprocessed sugar, lay on the bed of sugar tank in sugarcane processing mill (Olbrich., 2006). It is necessary to reduce the production cost in order to use the FOS as a feed additive extensively. Molasses can be used as an alternatives source replacing sucrose since pure sucrose is an expensive source for feed grade FOS production. Some researchers had come out with molasses as a substrate for the production of FOS. Shin et al., (2003) had reported that molasses can be used as a substrate in the FOS production by *A. pullulans.* The total FOS formed after 24 h incubation was 166 g/L from molasses which accounted about 46% of the sucrose as a substrate in molasses (Shin et al., 2003). FOS can be produced practically from molasses as a cheap source replacing sucrose although the enzyme reaction is inhibited to some extent by other sugars such as glucose and fructose in molasses.

2.6 FERMENTATION

Fermentation is one of the oldest and most economical methods of producing and preserving foods. Natural fermentation has already been used for ages to increase the shelf life of various food materials. This process has resulted in a number of traditional food products such as the dairy products cheese, butter, buttermilk and yoghurt; fermented meat, plants and fruits such as sausages, silage, sauerkraut, olives and grapes; and finally fermented cereal products such as bread and beer (Caplice and Fitzgerald, 1999). Indigenous fermented foods were developed through traditional or village art methodologies, which were preserved over the years, in order to maintain the uniqueness and identity of these foods. Fermented foods are essential components of the diet in a number of developing countries, and are consumed either as main dishes or as condiments (Sangeetha et al., 2005). They are prepared from both plant and animal materials, using processes in which microorganisms play an active role in the physical, nutritional and

organoleptic modification of the starting material. Due to the lack of scientific and technological, fermented foods are generally evaluated on the basis of qualitative attributes such as odor and flavor (Valyasevi and Rolle, 2000).

Biotechnological innovations have greatly assisted in industrializing the production of certain indigenous fermented foods. Indonesian tempe and oriental soy sauce are well known examples of indigenous fermented foods that have been industrialized and marketed globally (Valyasevi and Rolle, 2000). Nevertheless, the majority of fermented foods are produced using traditional methodologies at both the cottage and small scale levels in developing countries. In Africa, most of food fermentations are carried out on a household level, and are still conducted as spontaneous processes (Valyasevi and Rolle, 2000).

Those best adapted to the conditions during the fermentation process will eventually dominate. Initiation of a spontaneous process takes a relatively long time, with a high risk for failure. Failure of fermentation processes can result in spoilage and the survival of pathogens, thereby creating unexpected health risks in food products (Holzapfel, 2000). Thus, from both a hygiene and safety point of view, the use of starter cultures is recommended, as it would lead to a rapid acidification of the product and thus inhibit the growth of spoilage and pathogenic bacteria, and to a product with consistent quality (Holzapfel, 2000).

2.7 PREVIOUS TECHNIQUE TO PURIFY FRUCTOSYLTRANSFERASE

The properties of microbial FTase are different in each other microorganism and the culture medium composition especially the carbon sources which can play as an inducer role (Maiorano et al., 2008). Fructosyltransfering enzyme has been purified and characterized from higher plant such as asparagus (Shiomi et al., 1982), onion (Fujishima et al., 2004), Jerusalem artichoke (Koops and Jonker, 1994) and from different micro-fungi such as *Aspergillus* sp. (Sanchez et al., 2008), *Bacillus macerans* (Park et al., 2001), *Aureobasidium pullulans* (Lateef et al., 2006) and *Candida utilis* (Chavez et al., 1997). Although there are differ in unit structure, molecular weight, degree of glycosylation,

chemical susceptibility and substrate specificity, both hydrolytic and transfer activity are displayed which limits the fructooligosaccharides production to the use of high sucrose concentration (Maiorano et al., 2008).

Research has been shown several of sources have been used to purify and characterize the enzyme fructosyltransferase. There are many sources fungi, bacteria or plant that the enzyme can be isolated from which is *Aureobasidium pullulans* (Shin et al, 2003), *Aspergillus niger* (L'Hocine, 2000), *Bacillus macerans* (Park, 2001), *Aspergillus* sp. (Sanchez, 2008), *Aspergillus japonicus* (Chen et al., 2010) sugar beet leaves (Yun, 1996), lettuce (Yun, 1996). There is several technique of purification to characterize the enzyme used by researchers. Park (2001) stated that all of operation for enzyme purification was carried out at 4°C and centrifugation was conducted at 6000g for 15 minutes. Park (2001) has used 4 steps in order to purify the enzyme which is concentrated the crude enzyme solution by ammonium sulfate precipitation, then dialysis concentrated once again and finally analyzed the resulting enzyme solution by Fast Performance Liquid Chromatography (FPLC). L'Hocine (2000) stated 6 steps to purify the enzyme which is enzyme extraction, ammonium sulphate precipitation, and ion exchange chromatography on DEAE-sephadex A-25, gel filtration on Sepharose 6B, gel filtration on sephacryl S-200 and lastly affinity chromatography on ConA-sepharose 4B.

Since different result will be obtained when use different source, microorganism and strain, there is a need to have purification method and explore the research. (Lateef et al., 2006) found that the intracellular FTase of a novel strain of *Aureobasidium pullulans* capable of producing 59% (w/v) of FOS within 9 hours of reaction time. The other researcher L'Hocine (2000) has found that by using *Aspergillus niger* AS0023, the FOS produced by this strain is 54% (w/v). The production of FOS is depending on the enzyme activity which is depending on the medium condition. Sanchez (2008) has studied that the transfructosylating activity found at 60°C, pH 5.0 and at sucrose initial concentration was higher than 55 %(w/v).

2.8 GLOBAL MARKET OF FRUCTOOLIGOSACCHARIDES

Today's consumer hold high standard in choosing their nutrition. They demand food that taste great, calorie-reduced and the food that can make the better health to its consumer. FOS is one of the sources that can fulfill all these consideration. A lot of attention is being paid to dietary carbohydrates, especially oligosaccharides, in particular, fructooligosaccharides (FOS). The possible health benefits associated with the consumption of these compounds have led to their increased popularity as food ingredients and they are also being promoted as alternative sweeteners for diabetic formulations. In Europe, daily consumption of FOS has been estimated to be 3-11 g while 1-4 g has been estimated in US. The most common sources of FOS are wheat, honey, onion, garlic and banana (Flamm, Glinsmann, Kritchevsky, Prosky, & Roberfroid, 2001). FOS was first introduced into the market as foodstuff by Meiji Seika Co. in Japan during 1984 (Yun, 1996)

The concepts in nutrition have changed in the recent years. In the past, nutrition was chosen according to their ingredient that may cause bad effect to health. Presently, instead of choosing food according to their ingredient, the focus is change to the use of foods that promote a state of well-being, better health and reduction the risk of unwanted disease (Singh et al., 2010).

The global market of functional food is estimated up to 33 billion US dollars (Sangeetha et al., 2005). In US, the market is differentiated as functional foods with special health while in the Japan, there are specific health related food category called FOSHU (Food of Specified Health Used). An estimated market value of FOSHU labeled products was 2 billion US dollars in year 2000 with an estimated turnover of 14 billion US dollars (Sangeetha et al., 2005). The European functional foods market contributes to 4-8 billion US dollars (Sangeetha et al., 2005). The possible health benefits associates with FOS have led to their increased popularity as food ingredients and they are also being promoted as alternative sweeteners for diabetic formulations (Sangeetha et al., 2005).

2.9 EXPERIMENTAL DESIGN USING RESPONSE SURFACE METHODOLOGY

Response surface methodology (RSM) is an effective optimization tool wherein many factors and their interactions affect the response can be identified with fewer experimental trials. RSM mainly includes central composite design (CCD), Box Behnken design, one factor design, D-optimal design, user-defined design and historical data design. RSM had been widely used in various fields ranging from food process operations including extrusion (Karunanithy and Muthukumarappan, 2011).

Response surface methodology (RSM) is a statistical method that uses quantitative data from appropriate experiments to determine regression model equations and operating conditions. RSM is a collection of mathematical and statistical techniques for modeling and analysis of problems in which a response of interest is influenced by several variables (Karunanithy and Muthukumarappan, 2011). A standard RSM design called a central composite design (CCD) was applied in this work to study the variables of the FTase enzyme activity. This method was suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. Generally, CCD consists of a 2^n factorial runs with $2n$ axial runs and n_c center runs (six replicates) (Tan et. al., 2007).

Design expert version 6.0.6 software was used in this study to develop statistical analysis of the model and then was performed to evaluate the analysis of variance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 CHEMICALS AND EQUIPMENTS

The chemicals and equipments used in this study is of analytical grades, and they are summarizes in Table 3.1 and Table 3.2.

Table 3.1: List of equipment used

Equipment	**Brand**	**Principal Used**
Rotary shaker	Infors Ht	Fermentation
Refrigerated centrifuge	Eppendorf	Fermentation
Shaking water bath	BS-21	Enzyme assay
UV-Vis Spectrophotometer	U-1800	Analysis
Electrophoresis	Genei	Analysis
Peristaltic pump	Watson and Marlow	Purification

Table 3.2: List of chemical used

Chemical	**Supplier**	**Principal used**
Sucrose	Sigma, Aldrich	Fermentation
Yeast extract	Sigma, Aldrich	Fermentation
KH_2PO_4(monosodium phosphate)	Sigma, Aldrich	Fermentation
$NaNO_3$(Sodium nitrate)	Sigma, Aldrich	Fermentation
K_2HPO_4(Disodium phosphate)	Sigma, Aldrich	Fermentation
Sodium chloride(NaCl)	Sigma, Aldrich	Fermentation
$Mg.SO_4.7H_2O$ (magnesium sulphate heptahydrate)	Sigma, Aldrich	Fermentation
Sodium Hydroxide(NaOH)	Sigma, Aldrich	Analysis
Sodium Citrate Dihydrate	Sigma, Aldrich	Analysis
Acetonitrile	Sigma, Aldrich	Analysis
Ammonium sulphate	Sigma, Aldrich	Analysis
Phosphoric acid	Sigma, Aldrich	Analysis
Ovalbumin	Sigma, Aldrich	Standard
Carbonic anhydrase	Sigma, Aldrich	Standard
Bovine albumin	Sigma, Aldrich	Standard
Phosphorylase b	Sigma, Aldrich	Standard
Alcohol dehydrogenase	Sigma, Aldrich	Standard

3.2 OVERVIEW OF METHODOLOGY

Figure 3.1 summarizes the overview through of this entire study which is from the preparation of experiments until report writing.

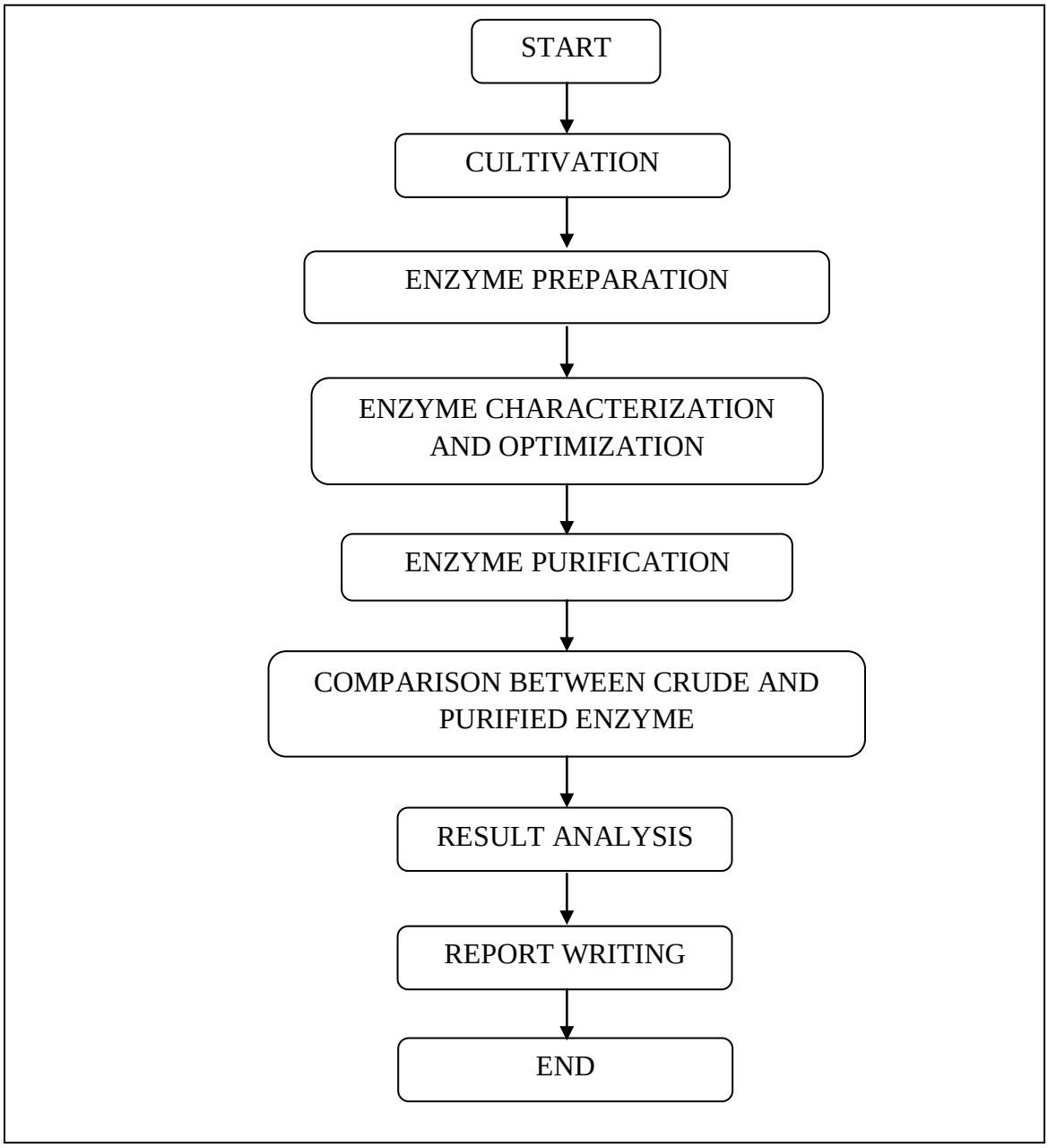


Figure 3.1: Overview of methodology

3.3 CULTURE METHOD

3.3.1 Agar Plate Culture

Aspergillus niger was aseptically transferred onto a plate containing sterile potato dextrose agar (PDA). The strain was subculture once in 2 weeks and incubated at 30 °C for 3 days or 72 hours. Then, the stock cultures were sealed and stored in refrigerator at 4 °C until further required usage.

3.3.2 Inoculum Preparation

The 3-days old strains were then was transferred into inoculum medium. The inoculum medium consists of 1% sucrose and 0.2% yeast extract at pH 5.5. The mycelia mat was formed by punched onto the agar plate using a sterile test tube to obtain a round disk of 0.5 cm in diameter. About two disks were put into 100 mL of inoculum medium in 250 mL shake flask. Then, the flask was incubated for 24 h at 30°C on rotary shaker at 250 rpm (Noraziah Abu Yazid, 2010). In order to get same growth rate in all shake flasks, the inoculum must be standardized which is the number of cell must be equal by using cell counting. Figure 3.2 illustrated inoculum that has been prepared.

Figure 3.2: Inoculum of *Aspergillus niger* after incubate for 24 hours

3.3.3 Fermentation of *Aspergillus niger*

A 24-hour old inoculum is transferred into 100 mL, pH 5.5 of fermentation medium consists of 17.5% w/v sucrose, 1% w/v peptone, 0.5% w/v yeast extract, 0.1% $MgSO_4.7H_2O$ and KH_2PO_4). By using incubator shaker at speed 250 rpm, the culture was incubated for 24h at 30±1 °C. Incubator shaker was shown in the Figure 3.3

Figure 3.3: Incubator Shaker Infors Ht

3.4 ENZYME ASSAY

3.4.1 Determination of FTase activity

At the end of incubation, 30 mL of the culture broth was centrifuged by refrigerated centrifuge for 15 minutes at 10 000 rpm and at 4°C for the extracellular enzyme source. The supernatant was taken as crude extracellular FTase. The activity of the enzyme was then determined. One unit (1U) defined as the amount of enzyme activity required to produce 1 µmol of glucose/min (Shin et al., 2003). By incubating 250 µL of enzyme with 750 µL of molasses, the FTase activity of the enzyme preparation was assayed. This is done in 0.1M citrate buffer (pH 5.0) at 55°C for 1 h in shaking water bath as shown in Figure 3.4 at 100 rpm to let the transfructosylation to occur. The reaction was stopped by keeping the reaction mixture in boiling water for 15 min (Lateef et al., 2006). FTase activity was determined as the amount of enzyme required to liberate 1 µmol of glucose under specified condition (Lateef et al., 2006). To calculate the enzyme activity based on the sugar

production, standard glucose calibration curve as shown in Appendix A1 was used. From Figure A1 (Appendix A1), the coefficient R^2 was 0.992 and the equation was y=2.761x + 0.062; where x-axis is a glucose production and y-axis is an absorbance of the UV VIS at 540 nm. This equation will be used to calculate the enzyme activity. The enzyme reaction was terminated by dipping the reaction mixture in boiling water bath or at 100 ℃ for 15 minutes (L'Hocine et al., 2000).

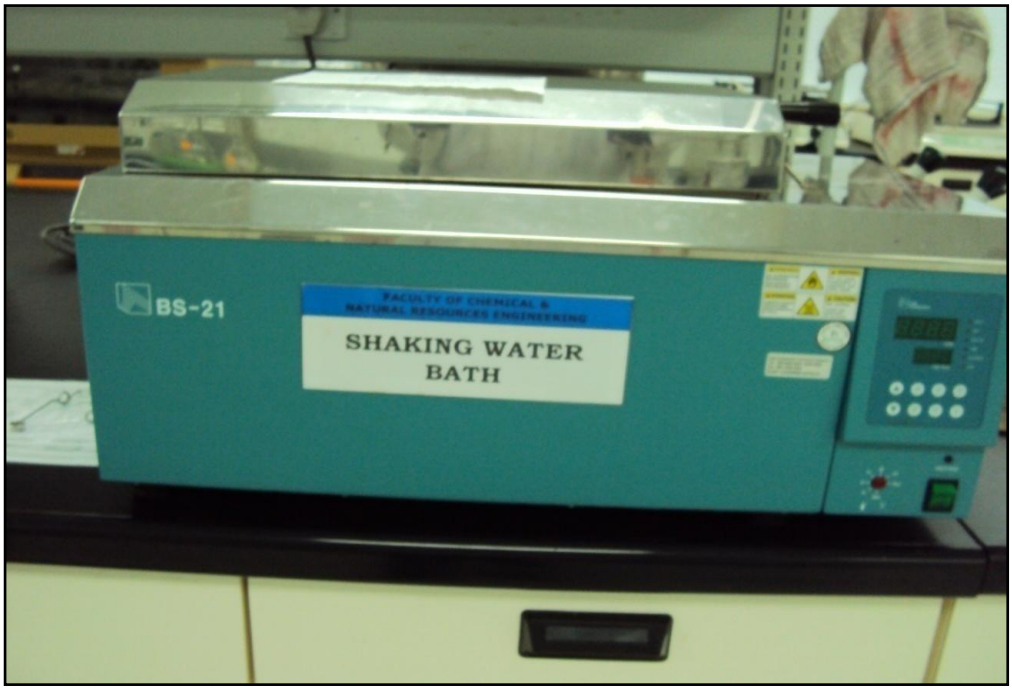


Figure 3.4: Shaking water bath BS-21

3.4.2 Determination of sucrose consumption

By using dinitrosalicyclic (DNS) method for glucose analysis, sucrose consumption can be determined. In this method, sucrose was hydrolyzed in an acid solution to yield glucose and fructose. 20 µL of concentrated HCl solution was added into 1 mL of supernatant and the reaction was allowed at 90 ℃ for 5 minutes. It is because of DNS method only can be applied in an alkaline condition; hence the KOH solution was added to neutralize the acid in order to develop the red brown color which represented the presence

of reducing sugar (Miller, 1959). DNS reagent was added into each test tube and dipped into boiling water for 5 minutes. The samples were then measured by using UV-Vis Spectrophotometer as shown in Figure 3.5. The standard calibration curve (Appendix A2) was generated in order to correlate the absorbance to the sucrose concentration.

Figure 3.5: U-1800 Ultraviolet Visible Spectroscopy (UV-Vis)

3.4.3 Determination of Biomass

The samples were withdrawn from the fermentation broth at every 8 hours interval for 48 hours. The sample was centrifuge at 10 000 rpm for 15 minutes at 4 °C. This was done to separate the biomass and the supernatant. The biomass was filtered on Whatmann No. 1 filter paper and washed several times using distilled water in order to remove media residues. The washed biomass was dried at 80 °C for 24 h to get the constant biomass weight. Figure 3.6 show the biomass obtained from broth fermentation

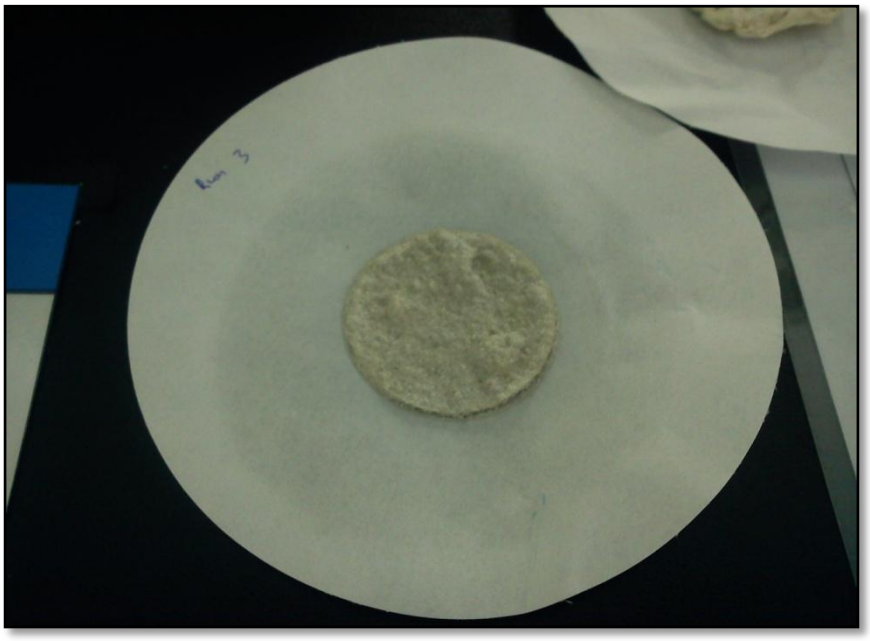


Figure 3.6: Biomass obtained from broth fermentation

3.5 DETERMINATION OF GLUCOSE CONCENTRATION BY USING DINITROSALICYCLIC (DNS) COLORIMETRIC METHOD

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline oxidations:

$$Aldehyde\ \ group \xrightarrow{oxidation} carboxyl\ \ group \quad (1)$$

$$3,5-dinitrosalicylic\ \ acid \xrightarrow{reduction} 3-a\min o,5-nitrosalicylic\ \ acid \quad (2)$$

Due to the dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen (Miller, 1959).

3.6 OVERVIEW OF THE PURIFICATION METHOD

Figure 3.7 summarizes the 5 steps of purification of enzyme.

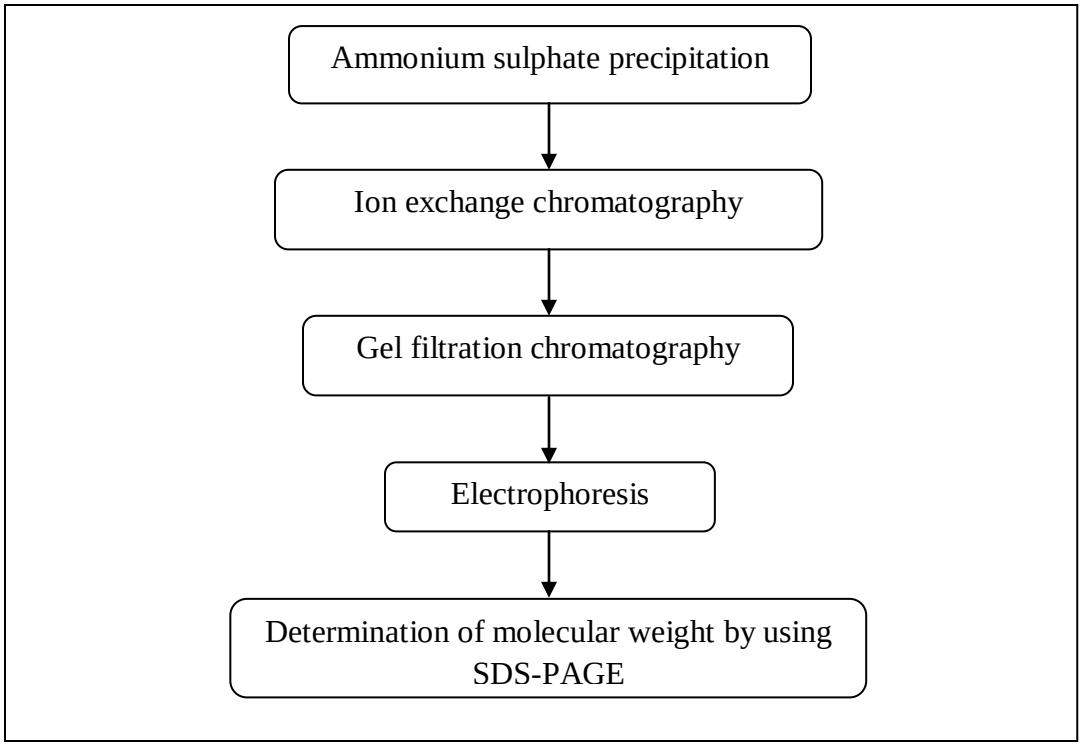


Figure 3.7: Overview of the purification method

3.6.1 Purification of Enzyme

There are 5 steps in order to purify the enzyme (Lateef et al., 2006):

3.6.1.1 Ammonium sulfate precipitation

The gradual fractionation of protein was started from 0%-30%, 30%-60% and finally 60%-80%. At each stage, analytical grade ammonium sulfates were slowly added to the crude enzyme with continuous stirring on a magnetic stirrer. The temperature of medium must be maintained below 10°C. At the end of each

step of fractionation, the mixture is stored at 4°C for 2h and then it was centrifuged at 12000 rpm for 20 minutes. The precipitate is then redissolved in a minimal amount of phosphate buffer (0.2 M, pH 7.0). Both precipitate and filtrate were assayed for FTase activity and the precipitation with high enzyme activity were pooled and dialyzed against changes of phosphate buffer to remove the ammonium salts. Dialysis was carried out using a cellulose dialysis membrane (Lateef et al., 2006).

3.6.1.2 Ion-exchange chromatography

The dialyzed sample (4.0mL) was loaded onto the column diethylaminoethyl (DEAE) cellulose and eluted with phosphate buffer (20mM, pH 7.0). Adsorbed protein was eluted with a linear gradient of 0.1-0.5 M NaCl at a flow rate of 10m/h using a peristaltic pump. Fractions were collected on a fraction collector and it is assayed for FTase activity. The protein content was measured at the optical density (OD) of 280nm. The active fractions were pooled and lyophilized using a Heto Dry Winner then the lyophilized protein was resuspended in a minimal amount of phosphate buffer (20mM, pH 7.0) (Lateef et al., 2006).

3.6.1.3 Gel Filtration Chromatography

The lyophilized sample was dissolved in 1mL of phosphate buffer (pH 7.0) and loaded onto Sephadex G200 column. But before that, this column must be equilibrated with 20mM, pH 7.0 of phosphate buffer. At flow rate of 10mL/h, the proteins were eluted with the same buffer. The FTase activity and protein content of each fraction were determined and the active fraction were pooled, lyophilized and stored at 4°C (Lateef et al., 2006).

3.6.1.4 Electrophoresis

Electrophoresis analysis was carried out using a Genei electrophoresis kit. In this study, the technique used to stain the protein in the gel is silver staining technique (Lateef et al., 2006).

3.6.1.5 Determination of molecular weight

The mobility of each protein band was determined as the ratio of distance moved by each protein band to the distance moved by tracking dye (Lateef et al., 2006) as shown in the Eq. 3:

$$mobility\ of\ protein = \frac{\text{distance moved by each protein band}}{\text{distance moved by tracking dye}} \quad (3)$$

The logarithm of molecular weight was plotted against the mobility to obtain the regression equation. The molecular weight of the purified protein then obtained by interpolation.

3.7 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE is a technique that widely used in biochemistry, forensics, genetic and molecular biology. It is been used to separate proteins on the basis of polypeptide length and then their molecular weight can be estimated. It is anionic and the molecules have a net negative charge within a wide pH range. During SDS-PAGE, the negative charge on the SDS destroy most of the complex structure of proteins and all proteins migrate toward the positively charge electrode which is anode. SDS-PAGE was used to identify fraction that contain the targeted protein by separating the proteins according to their molecular weight. This was done first through gel electrophoresis of the collected sample and then loaded alongside known standards, and then allowed to run along the gel in order to separate based on molecular weight (Tsumori et al., 1984).

3.7.1 Molecular Structure of SDS

The application of SDS PAGE in industry is to evaluate genetic purity and diversity of several varieties It is the most common analytical technique used to separate the characterize proteins. The acrylamide and bisacrylamide solution is used as polymerized. The acrylamide forms a linear polymers and bisacrylamide introduces crosslinks between polyacrylamide chains (Tsumori et al., 1984).

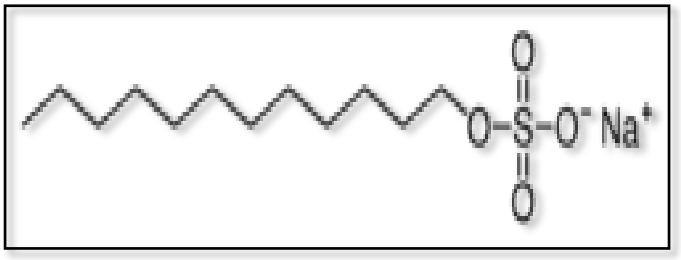


Figure 3.8: Molecular Structure of SDS

3.7.2 SDS-PAGE Preparation

10% separating gel and 5% stacking gel solution was prepared by mixing of various stock solutions that are necessary for all components of both gels. The mold is set up which includes two glass plates, with appropriate spacer, inserted into a clamp, which itself is inserted onto a stand that seals all side of the plates except the top. The mold was checked and make sure there are no leaking. First, the separating gel is poured into the mold as actual separation proposes. It consists of a higher amount of acrylamide. Then, a layer of water is applied on top as the running gel polymerizes. The application of a water layer is to remove air bubble which may have formed during pouring as well as to create a level surface onto which the stacking gel can be applied (Tsumori et al., 1984). The process is nearly identical for the stacking gel, which differs from the separating gel in composition, except the comb is inserted during polymerization in order to create the wells where the sample will be applied. After polymerization, detach the casting base and place the slab in the chamber of electrophoresis system that is filled with electrophoresis buffer. Conduct the

electrophoresis at a voltage of 150 V for about 60 min (Tsumori et al., 1984). Figure 3.9 summarizes the step on how to prepare SDS-PAGE.

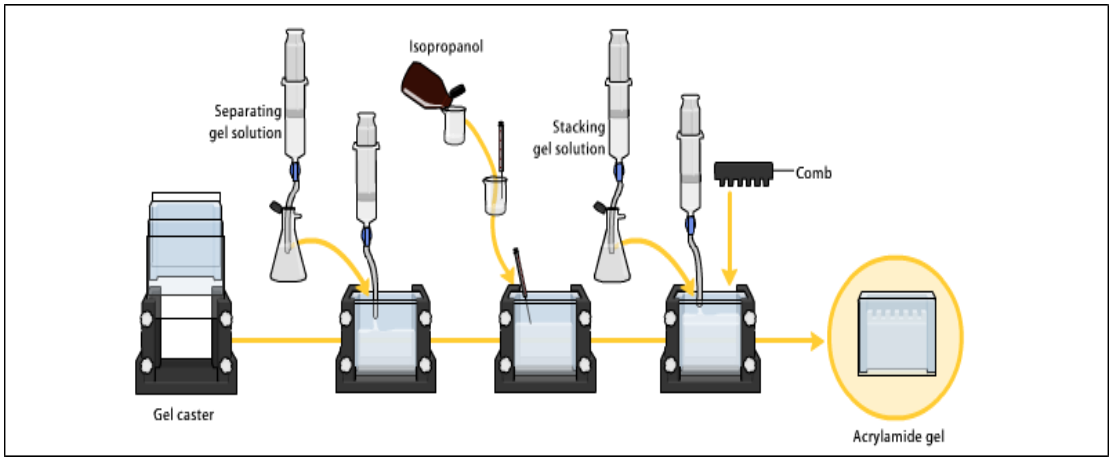


Figure 3.9: Gel Preparation for SDS-PAGE

CHAPTER 4

RESULTS AND DISCUSSION

4.1 FERMENTATION STUDIES

In shake flask experiments, it was conducted at 30 °C for 48 hours to determine the optimum parameter condition. It is done by changing one independent variable while fixing others at a certain level. Process parameter involve in this study are pH, substrate concentration and temperature. To optimize the production of FTase, a response surface methodology with central composite design (CCD) is been used in this study.

4.1.1 Effect of Initial pH

FTase production is influenced by pH of the culture medium. As Dhake and Patil (2005) carried out in their study, they stated that initial pH play key role in enzyme production and in utilization of media constituents and growth of the microorganism. To investigate the effect of pH on bioconversion of sucrose to glucose, it would be important to keep this parameter constant at the beginning of the fermentation period. Table 4.1 summarizes the crude, purified enzyme activity and biomass obtained by using different pH. Figure 4.1 and Figure 4.2 show the crude and purified FTase activity and biomass produced by *A. niger*. The highest purified FTase activity was observed at pH 5.5 which correspond to 128 U/mL while crude enzyme activity is 104.7 U/mL after 48 h fermentation period. At pH 4 to 5, the crude FTase activities were exhibited to be 75.8 U/mL and 81.3 U/mL while for purified enzyme is 78.4 U/mL and 92.1 U/mL respectively which is lower than activity at pH 5.5. Similar effect of initial pH was also occurred for the

sucrose-to-glucose bioconversion by the FTase of *Aspergillus oryzae* CFR 202 in solid state fermentation using agricultural by-products as a substrate (Sangeetha et al., 2003). The activity of enzyme decrease drastically at pH more than 6.0 and almost no activity when the condition of medium in alkaline condition. This shows that the FTase enzyme is active near the neutral environment with initial pH ranged from 5.5 to 6.5.

Table 4.1: Effect of pH towards crude and purified enzyme activity (U/mL) and biomass production (mg/L)

pH	**Temperature (°C)**	**Substrate Concentration (mg/L)**	**Crude Enzyme Activity (U/mL)**	**Purified Enzyme Activity (U/mL)**	**Biomass (Crude) (mg/L)**	**Biomass (Purified) (mg/L)**
4	30	10	70.8	71.5	4.376	4.438
4.5	30	10	75.8	78.4	4.693	4.853
5.0	30	10	81.3	92.1	5.438	5.637
5.5	30	10	104.7	128	8.316	8.526
6.0	30	10	96.5	105.6	6.725	7.324
6.5	30	10	86.7	98.1	6.327	6.587
7.0	30	10	31.9	49	3.926	4.365

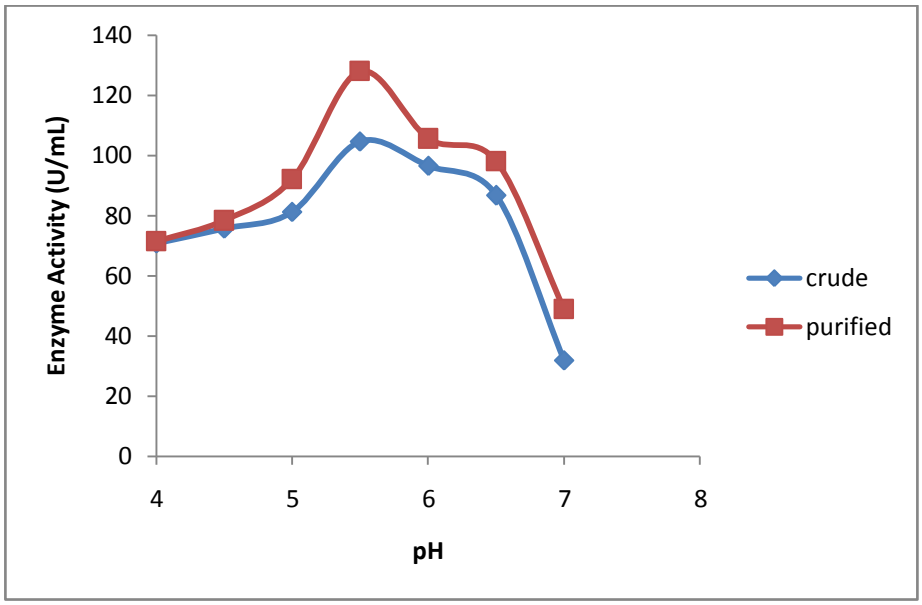


Figure 4.1: Effect of pH towards crude and purified enzyme activity

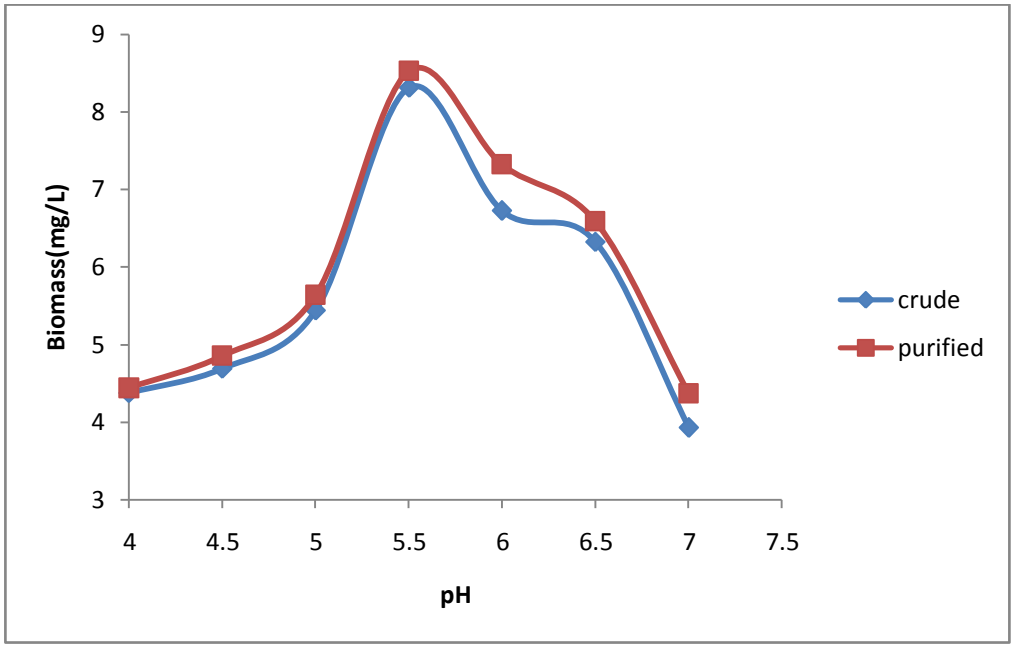


Figure 4.2: Effect of pH on biomass

4.1.2 Effect of initial substrate concentration

During the microbial fermentation, the carbon source is important to provide energy source and also to build the cellular material. In order to study the feasibility of molasses as a carbon source for FOS production by *A. niger*, different concentration of substrate were used. Table 4.2 summarizes the result by using different substrate concentration towards enzyme activity. As shown in Figure 4.3, the highest purified enzyme activity was observed at 25 mg/L of substrate concentration. As the FTase production is increased, the FOS yield will also increase (Nemukula et al., 2008). From Figure 4.3, we can see that further increase in substrate concentration showed the reduction of enzyme activity thus yield of FOS will decrease too. Increasing in substrate concentration may contribute to the substrate saturation for the enzyme FTase. Similar behavior has been reported by (L'Hocine et al., 2000) that increasing of sucrose concentration will cause the transfer reaction decrease slowly due to the water activity. The decreasing enzyme activity or transfructosylation reaction will result in low FOS yield. As shown in the Figure 4.4, the amount of biomass produced ranged from 4.987 mg/L to 5.983 mg/L after 48 hour of incubation period. From Figure 4.3 and 4.4, the enzyme activity increase when biomass increased. Dhake and Patil (2005) had reported that there was increase in biomass FTase production when sucrose concentration increases. This may be because of availability of substrate and an enhanced mass transfer effect.

Table 4.2: Effect of substrate concentration (g/L) towards crude and purified enzyme activity and biomass production (mg/L)

pH	Temperature (°C)	Substrate Concentration (g/L)	Crude Enzyme Activity (U/mL)	Purified Enzyme Activity (U/mL)	Biomass (Crude) (mg/L)	Biomass (Purified) (mg/L)
5.5	30	10	72.6	82.6	4.376	5.023
5.5	30	15	78.6	94.6	4.693	5.684
5.5	30	20	84.3	103.5	5.438	6.231
5.5	30	25	96.5	112.4	8.316	8.235
5.5	30	30	89.2	95.6	6.725	7.236
5.5	30	35	84.7	92	6.327	6.538
5.5	30	40	78.1	81	3.926	6.037

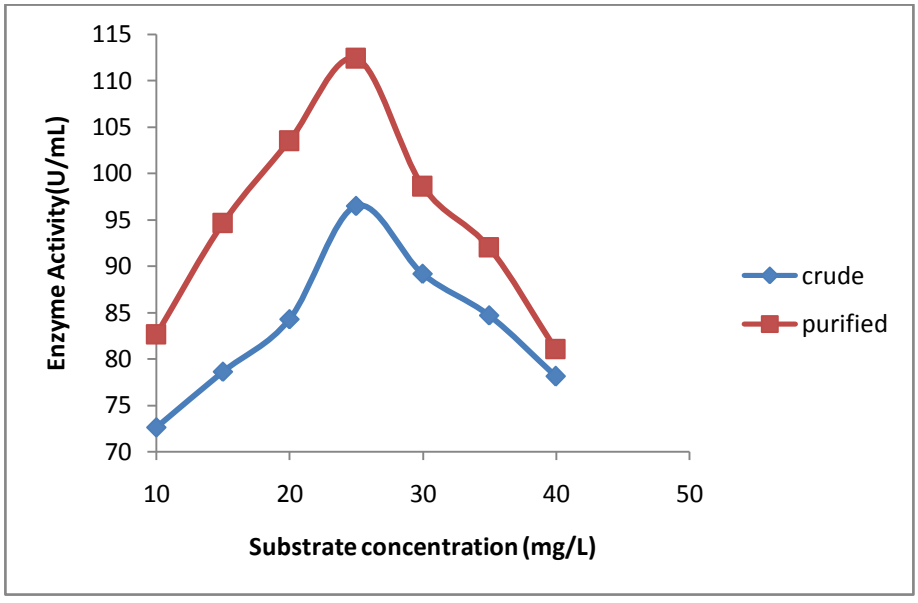


Figure 4.3: Effect of substrate concentration towards crude and purified enzyme activity

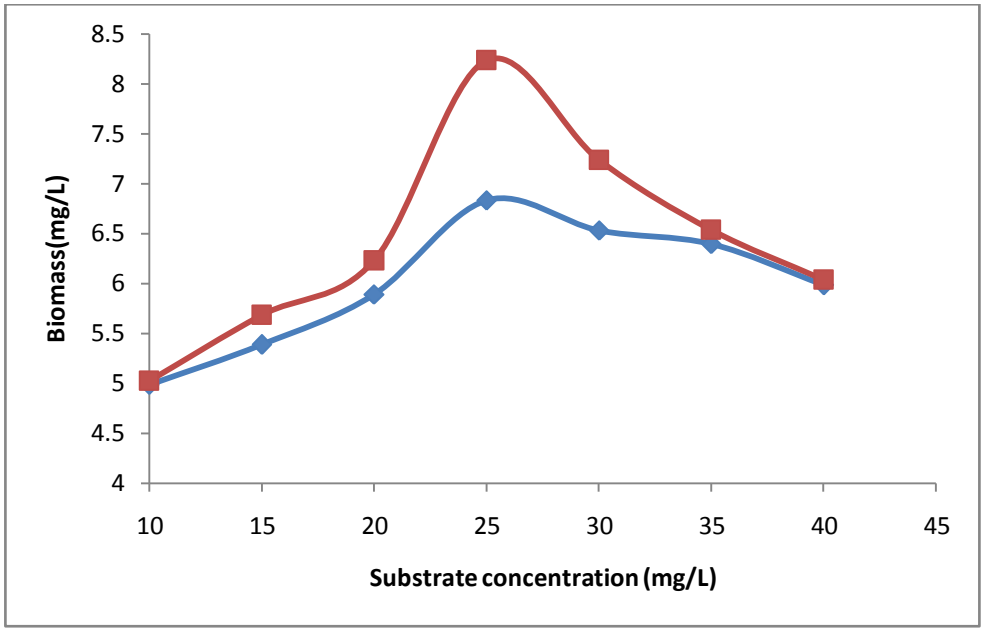


Figure 4.4: Effect of substrate concentration towards biomass production

4.1.3 Effect of temperature

The FTase activity of the enzyme was determined at the different temperature 50°C, 55 °C, 60 °C, 65 °C and 70 °C. Table 4.3 summarizes the result by using different temperature towards enzyme activity.

Table 4.3: Effect of temperature (°C) towards crude and purified enzyme activity and biomass production (mg/L)

pH	Temperature (°C)	Substrate Concentration (g/L)	Crude Enzyme Activity (U/mL)	Purified Enzyme Activity (U/mL)	Biomass Crude (mg/L)	Biomass Purified (mg/L)
5.5	40	10	53.7	62.3	4.527	4.794
5.5	45	10	64.9	75.2	5.017	5.386
5.5	50	10	87.3	94.3	5.619	6.326
5.5	55	10	108.5	125	7.281	8.234
5.5	60	10	98.2	100	6.973	7.069
5.5	65	10	89.4	94	6.402	6.215
5.5	70	10	73.6	82	4.765	4.987

From Figure 4.5, the optimal point of enzyme activity (affected by thermal) was observed at 55 ^{0}C. Further increment in temperature lead to decreasing of enzyme activity. As stated by Ghazi et al (2005), the enzyme will not be effective when the temperature is too high and then the transfructosylation reaction will be reduced. The enzyme at high temperature will undergoes irreversible denaturation which may be due to the permanent temperature-dependent unfolding (Ozimek et al., 2006). The FTase enzyme will not effectively produce the FOS in high yield. When the biomass production increase, FTase activity also increased. As shown in Figure 4.6, high production of biomass when using crude enzyme was observed at 55 °C with yield of 7.281 mg/L while for purified, biomass obtained was observed at 8.234 mg/L. . The biomass concentration profiles were similar to the FTase activity, showing that the enzyme production is related to the biomass concentration. Thus implying that a high biomass concentration may ensure a higher FTase production. Sanchez et al. (2008) also in agreement with this phenomenon as they indicated that under lower concentration of biomass, FTase production by *Aspergillus sp.* N74 was also low.

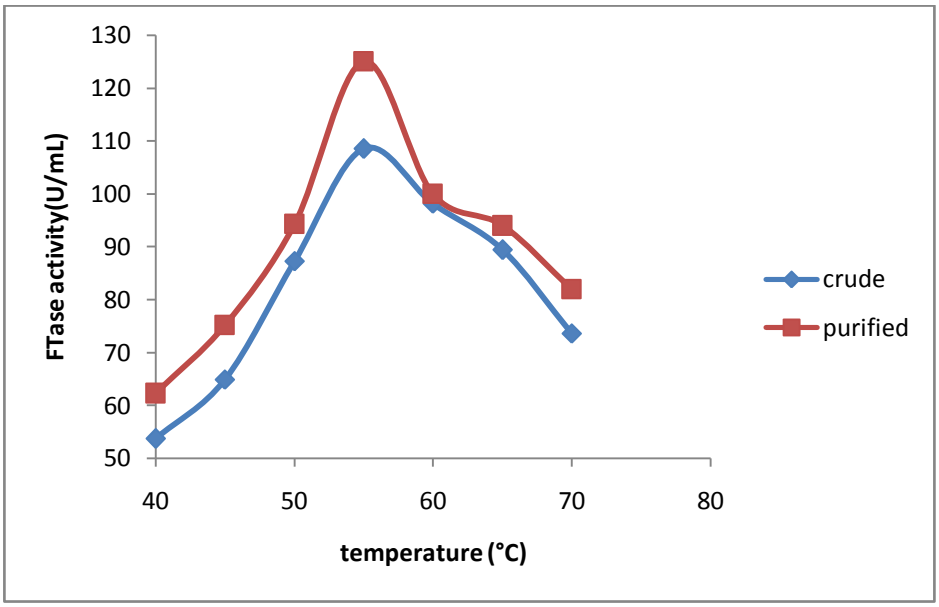


Figure 4.5: Effect of temperature on crude and purified enzyme activity

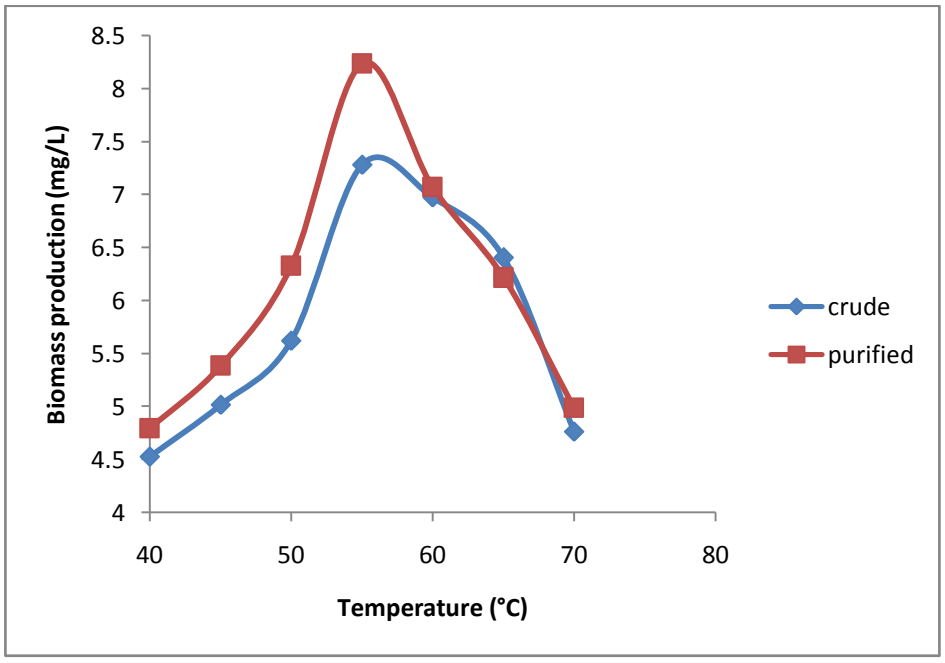


Figure 4.6: Effect of temperature (°C) towards biomass production (mg/L)

4.2 RESPONSE SURFACE METHODOLOGY (RSM) APPLICATION

A central composite design (CCD) was employed to reduce the total number of experiments needed to determine the best combination of parameters for optimization of the process. The statistical software Design Expert, version 6.0.6, Stat-Ease, Inc, Minneapolis, MN was used for the CCD and to analyze the experimental data obtained. Table 4.4 shows the experimental layout and results of CCD. There were 6 duplicates at the central coding condition with experimental condition; 55 °C, pH 6.0 and 20 g/L substrate concentration.

Table 4.4: Experimental design of three parameters variable (pH, substrate concentration and temperature) and crude enzyme activity as the response using central composite design (CCD)

			Factor Variables			Response
Standard	**Run**	**Block**	**Temperature (^{0}C)**	**pH**	**Concentration (mg/L)**	**Enzyme Activity (U/mL.min)**
1	16	1	50	5.0	10	90.0
2	1	1	60	5.0	10	71.7
3	10	1	50	7.0	10	59.3
4	3	1	60	7.0	10	48.3
5	11	1	50	5.0	30	148.3
6	14	1	60	5.0	30	141.7
7	19	1	50	7.0	30	108.0
8	4	1	60	7.0	30	50.6
9	9	1	45	6.0	20	90.0
10	13	1	65	5.5	20	135.0
11	18	1	55	4.0	20	95.8
12	6	1	55	5.5	20	125.0
13	7	1	55	6.0	30	123.3
14	2	1	55	6.0	40	60.5
15	5	1	55	6.0	20	89.4
16	17	1	55	6.0	20	103.0
17	20	1	55	6.0	20	116.7
18	12	1	55	6.0	20	120.0
19	8	1	55	6.0	20	123.3
20	15	1	55	6.0	20	106.7

For optimization, three variable conditions were optimized using RSM to study the response of enzyme activity towards pH, substrate concentration and temperature as shown in Table 4.6. In this experiment, there are 20 experiments. For pH, low value of pH is 5.0 and high value of pH is 7.0. For temperature, low value is 45 °C and high value is 65 °C. Summary of range and level are represented in Table 4.5.

Table 4.5: Experimental ranges and level of the independent variables

Independent variable	**Range and level**				
	-α	**-1**	**0**	**+1**	**+α**
pH	4	5	6	7	8
Substrate concentration(g/L)	5	10	20	30	40
Temperature	45	50	55	60	65

Table 4.6: Experimental design based on CCD used in this study

Run No.	**Independent values**					
	Coded values			**Experimental values**		
1	+1	-1	-1	60	5	10
2	0	0	+2	55	6	60
3	+1	+1	-1	60	7	10
4	+1	+2	+1	60	8	30
5	0	0	0	55	6	20
6	0	+2	0	55	5.5	20
7	0	0	+1	55	6	30
8	0	0	0	55	6	20
9	-2	0	0	45	6	20
10	-1	+1	-1	50	7	5
11	-1	-1	+1	50	5	30
12	0	0	0	55	6	20
13	+2	+2	0	65	5.5	20
14	1	-1	+1	60	5	30
15	0	0	0	55	6	20
16	-1	-1	-1	50	5	10
17	0	0	0	55	6	20
18	0	-2	0	55	4	20
19	-1	+1	+1	55	7	30
20	0	0	0	55	6	20

As can be inferred in Table 4.7, the computed *F test* and *Prob>F* were 3.74 and 0.0805 respectively, which implied that the model was highly significant with low probability. Results obtained adequately suggesting that the present mathematical model was in good prediction of the experimental results and as a matter of fact the terms in the model have a significant effect of the response. Moreover, the "lack of fit" value was found insignificant (Prob>F= 0.0805) which denoted that the model was desirably fit.

Fit summary output analysis indicated that the quadratic model was statistically significant to represent the enzyme activity response. The adequacy of a quadratic model was examined by *F test*, "*Prob>F*" and the determination coefficient R^2. In this case, the value of the determination coefficient for enzyme activity is 0.7709 indicate that a good agreement existed between the experimental and predicted value as well as depicting that 77.09 % of the variability in the response could be well explained by the model while 22.91 % of the total variation was poorly described by the model. The closer the R^2 is to 1, the stronger the model and the better it predicted the response (Noraziah, A.Y., 2011)

Table 4.7: Analysis of variance (ANOVA) for response quadratic model (partial some of square), response; crude enzyme activity (U/mL/min)

Source	Sum of Squares	DF	Mean Squares	F Value	Prob>F
Model	13307.24	9	1478.58	3.74	0.0259[a]
A	63.08	1	63.08	0.16	0.6981
B	3878.33	1	3878.33	9.80	0.0107
C	3510.83	1	3510.83	8.88	0.0138
A^2	4.64	1	4.64	0.012	0.9159
B^2	1538.07	1	1538.07	3.89	0.0769
C^2	4419.31	1	4419.31	11.17	0.0075
AB	406.73	1	406.73	1.03	0.3345
AC	150.51	1	150.51	0.38	0.5511
BC	746.91	1	746.91	1.89	0.1994
Residual	3955.80	10	395.58		
Lack of Fit	3149.90	5	629.98	3.91	0.0805[b]
Pure Error	805.89	5	161.18		
Cor Total	17263.04	19			
Std. Dev.	19.89		R^2	0.7709	
Mean	100.33		Adjusted R^2	0.5646	

Values of "prob>F" less than 0.0500 indicate model are significant.

[a] *significant*

[b] *not significant*

Final empirical model in terms of coded factors:

$$Enzyme\ activity = +113.71 - 2.02A - 20.58B + 19.61C - 0.43A^2 - 11.13B^2 - 19.16C^2 - 7.00AB - 4.34AC - 9.66BC \quad (4.1)$$

Final empirical model in terms of actual factors:

$$Enzyme\ activity = -982.61467 + 11.61519temperature + 209.40188pH + 20.19284substrate - 0.017083temperature^2 - 11.13395pH^2 - 0.19158substrate^2 - 1.40084(temperature)(pH) - 0.086750(temperature)(substrate) - 0.096625(pH)(substrate) \quad (4.2)$$

Equation 4.2 is an empirical model equation of mathematical correlation model that can be employed to predict and optimize the enzyme activity within the range of variable factors of this experiment. Analyses on normal probability plot of the residuals for crude enzyme activity shown by Figure 4.7 depicted nearly a straight line residuals distribution, which donating errors are evenly distributed and therefore support adequacy of the least-square fit, while results illustrated in Figure 4.8 revealed that the models proposed are distinctively adequate and reasonably free from any violation of the independence or constant variance assumption, as studentized residuals are equally tabulated within the red line of the x-axis.

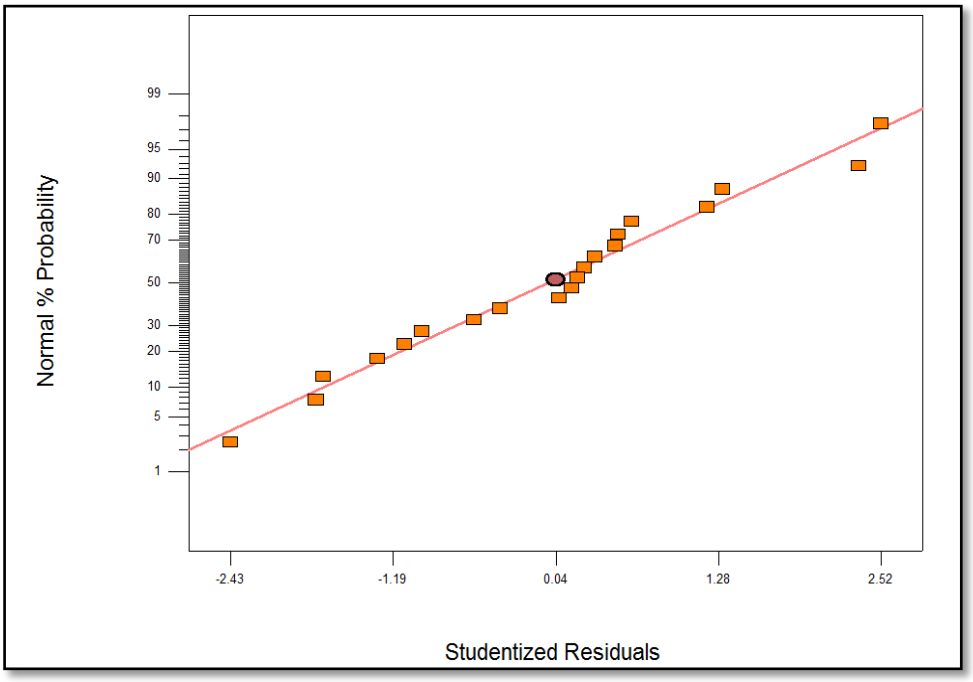


Figure 4.7: Normal probability plot of residuals for crude enzyme activity

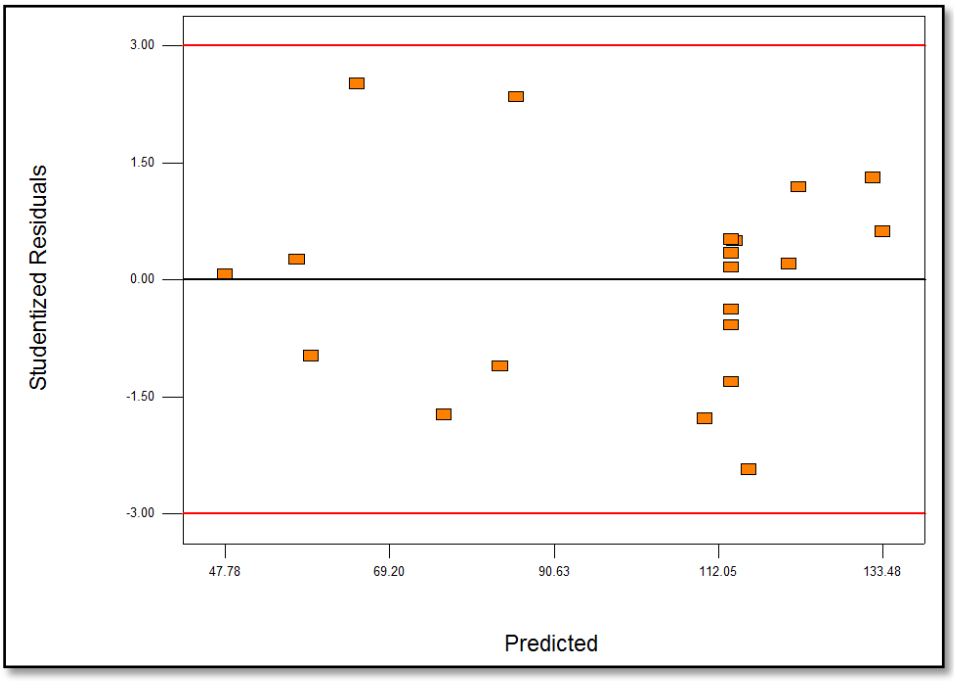


Figure 4.8: Plot of residual against predicted response of crude enzyme activity

The effect of pH, temperature and concentration of substrate process variables on enzyme activity was further analyzed using simulated three dimensional response surface and contour plots. From the graph interaction, the effect of pH and temperature on enzyme activity depicted in Figure 4.9 and Figure 4.10 shows that the activity of the crude FTase enzyme increased when pH change from 5 to 7 and as reaction temperature increased from 50 °C to 60 °C. In Figure 4.10, an inclined form of three dimensional plot inferred that interaction between pH and temperature were evident with significant linear correlations. Generally, the effect of pH on microbial growth may be attributed to the hydrogen (H^+) ion concentration. H^+ can be considered as substrate under the pH range 6.0 - 7.0 but act as inhibitor under acidic and alkaline pH. Additionally, the enzyme has almost no transfructosylating activity below pH 3.0 or above pH 10.0 (Sautour, 2003).

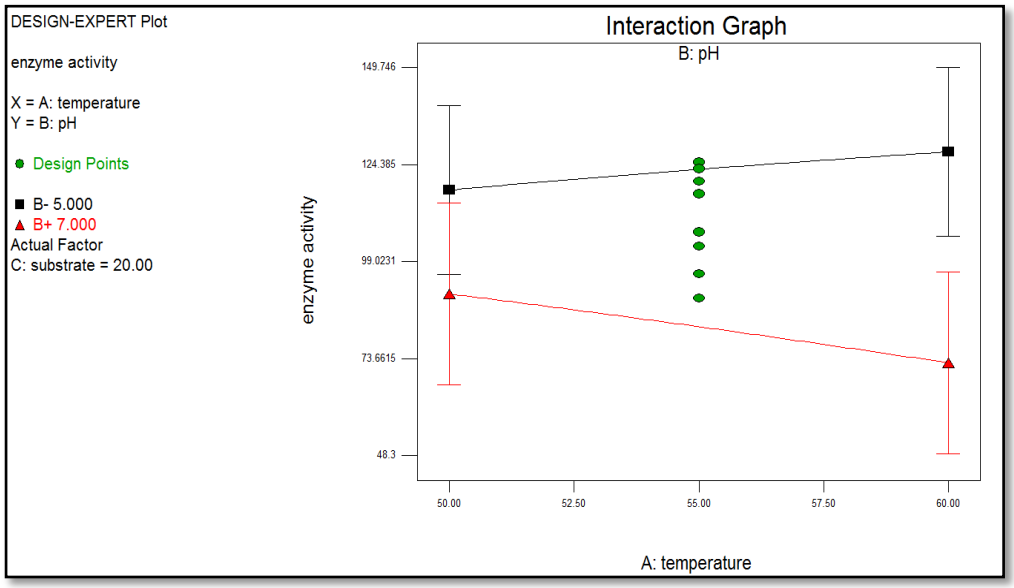


Figure 4.9: Interaction graph of crude enzyme activity from the model equation: effect of temperature (°C) and pH

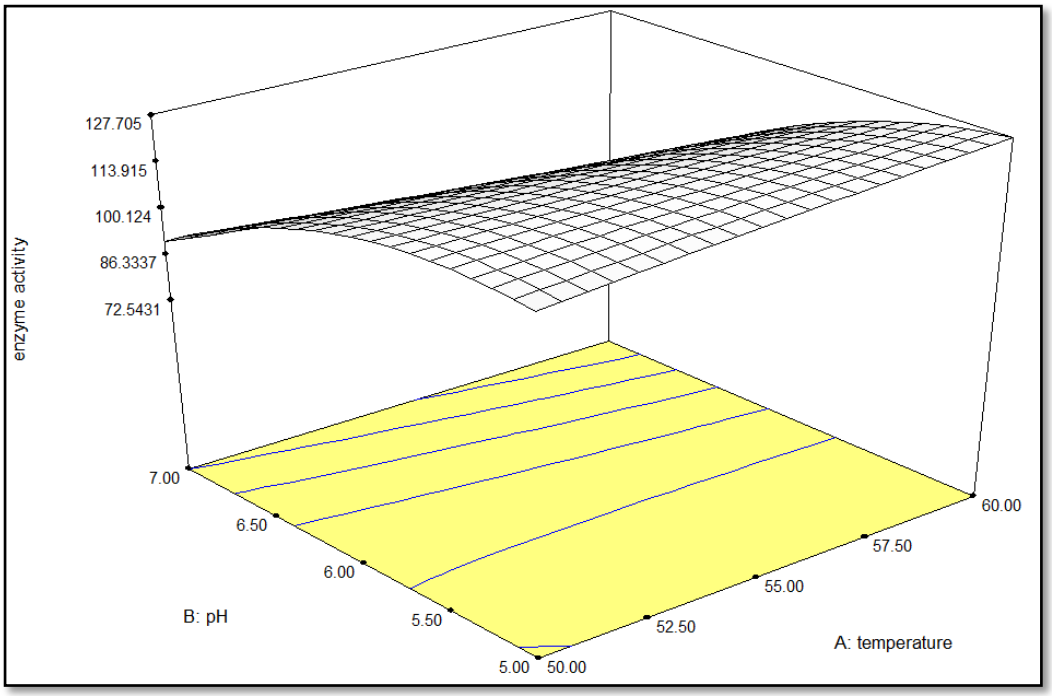


Figure 4.10: Three dimensional (3D) graph of crude enzyme activity from the model equation: effect of temperature and pH

The interaction graph (Figure 4.11) and 3D plots as shown in Figure 4.12 shows the effect of pH and substrate concentration on crude FTase enzyme activity are shown in Figure 4.12. The maximum enzyme activity of 125 U/mL is obtained when pH at 5.5 and temperature set at 55 ^{0}C respectively. It was clearly shown that the enzyme activity was increased from 117.737 U/mL at 50 ^{0}C to 127.705 U/mL at 60 ^{0}C when substrate concentration constantly maintained at 20%. The other site, the opposite result was observed with the decreasing of enzyme activity at 90.5914 U/mL at 50 ^{0}C to 72.5431U/mL at 60 ^{0}C. In the early, the enzyme activity of FTase was increased but when achieved at middle point, the enzyme activity become decreasing uniformly. It is because of when pH is 5.5, it promoted transfructosylation to occur. It is been stated by Nemukula et al (2008) that a pH of 5.6 promoted transfructosylation to occur and the temperature of 60 °C (Nemukula et al., 2008) is the optimum since it increased sucrose solubility and prevent microbial contamination. While in this study, the optimum temperature is observed at 55 °C.

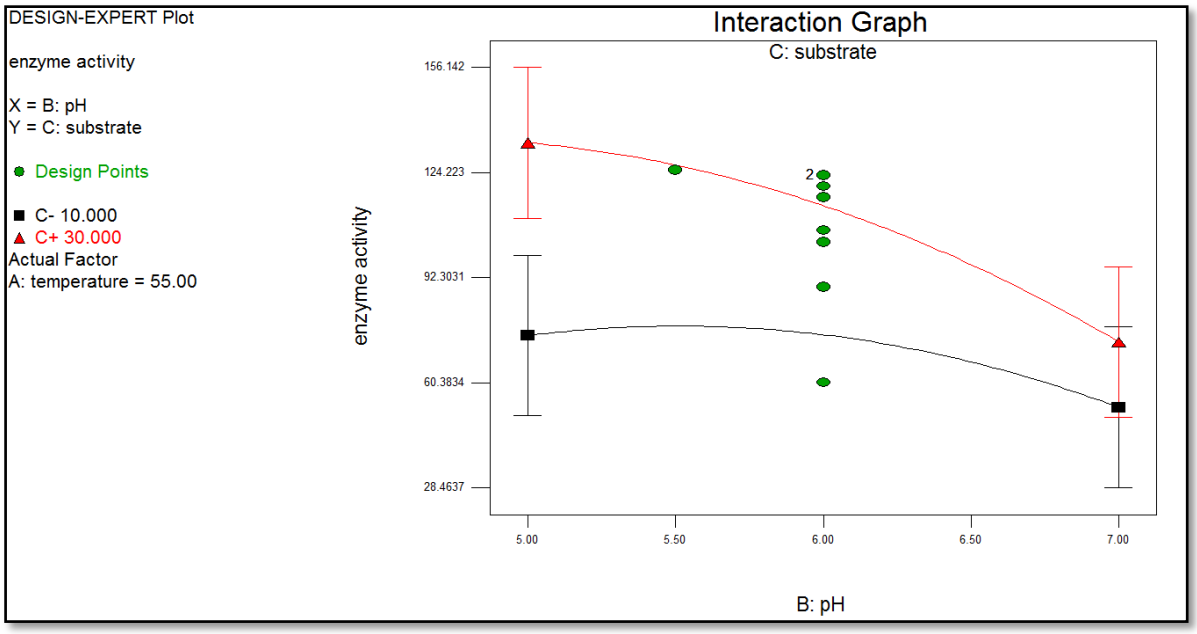


Figure 4.11: Interaction graph of crude enzyme activity from the model equation: effect of pH and substrate concentration (g/L)

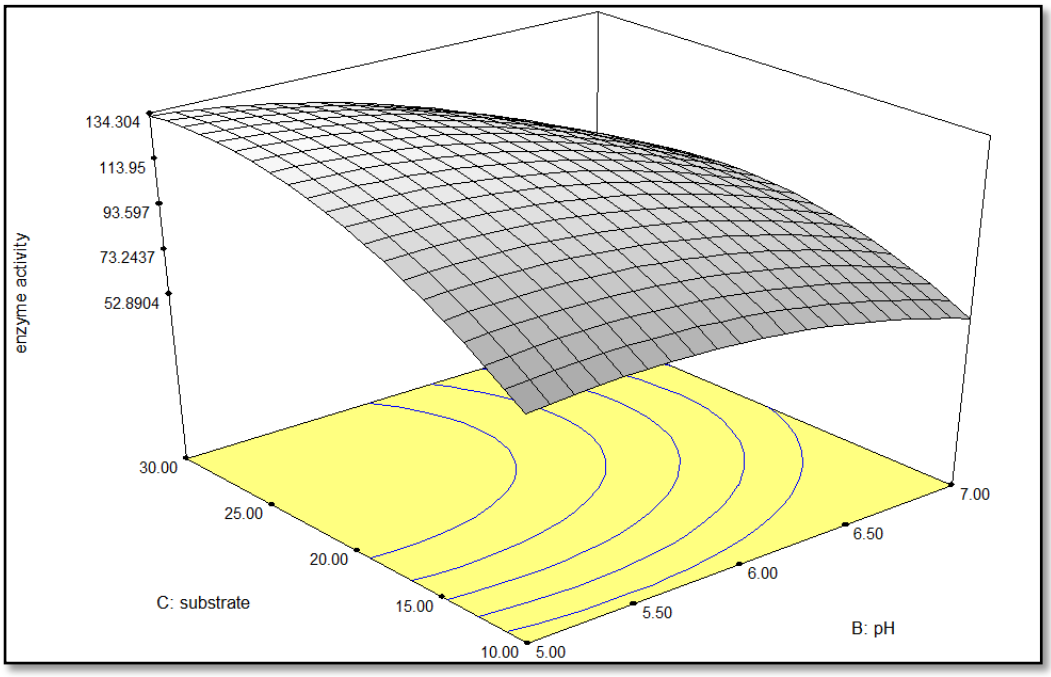


Figure 4.12: Three dimensional (3D) graph of crude enzyme activity from model equation: effect of pH and substrate concentration (pH 5.0 to 7.0 and substrate concentration 10 – 30 g/L)

Based on the interaction graph, Figure 4.13 and Figure 4.14 show that the activity of the purified FTase enzyme increased when pH change from 5 to 7 and as reaction temperature increased from 50 °C to 60 °C. Based on Figure 4.14, the maximum enzyme activity of 136.7 U/mL is obtained when pH at 5.5 and temperature set at 55 ^{0}C respectively.

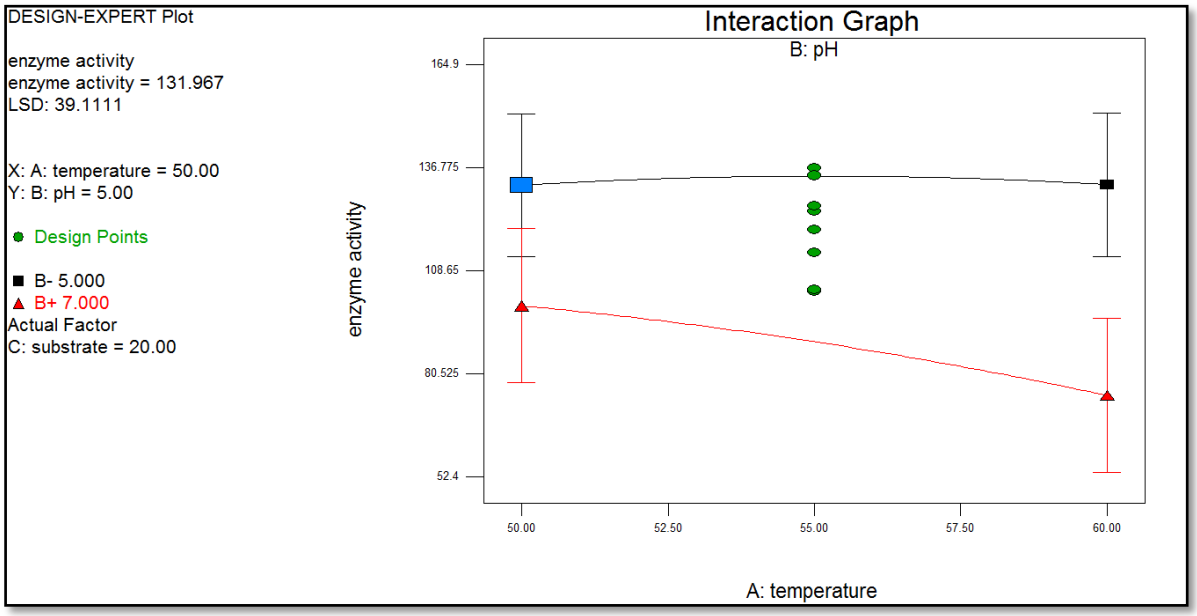


Figure 4.13: Interaction graph of purified enzyme activity from the model equation: effect of temperature (°C) and pH

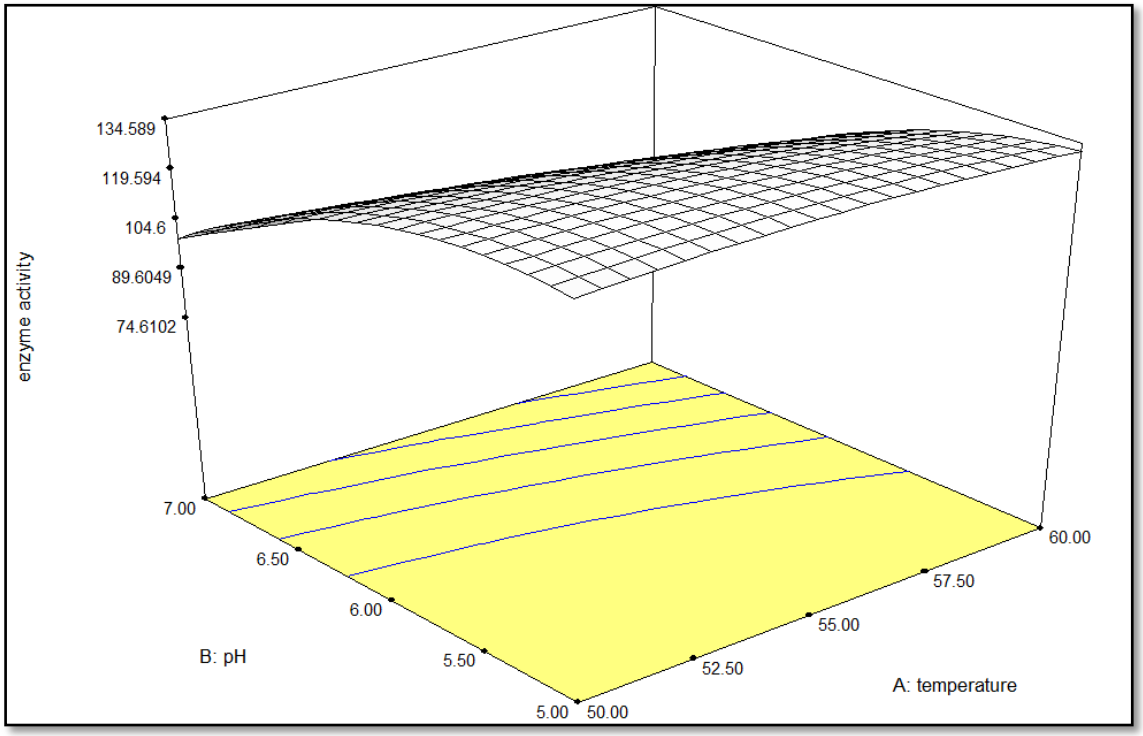


Figure 4.14: Three dimensional (3D) graph of purified enzyme activity from the model equation: effect of temperature and pH

From the interaction graph as shown in Figure 4.15 and 3D surface plot as shown in Figure 4.16, it was clearly shown that the enzyme activity was increased from 131.967 U/mL at 50 ^{0}C to 132.169 U/mL at 60 ^{0}C when substrate concentration constantly maintained at 20%. The other site, the opposite result was observed with the decreasing of enzyme activity at 99.0578 U/mL at 50 ^{0}C to 74.6102 U/mL at 60 ^{0}C. Yun (1996) had reported that the enzyme production only occurred when sucrose is present in culture medium which acts as carbon source. However, in this study the higher concentration of substrate (30-40 g/L) resulted in inhibition of cell growth which affected FTase activities. In can be concluded from the results, that the concentration of the limiting substrate sucrose had a significant effect on the FTase activities and cell growth

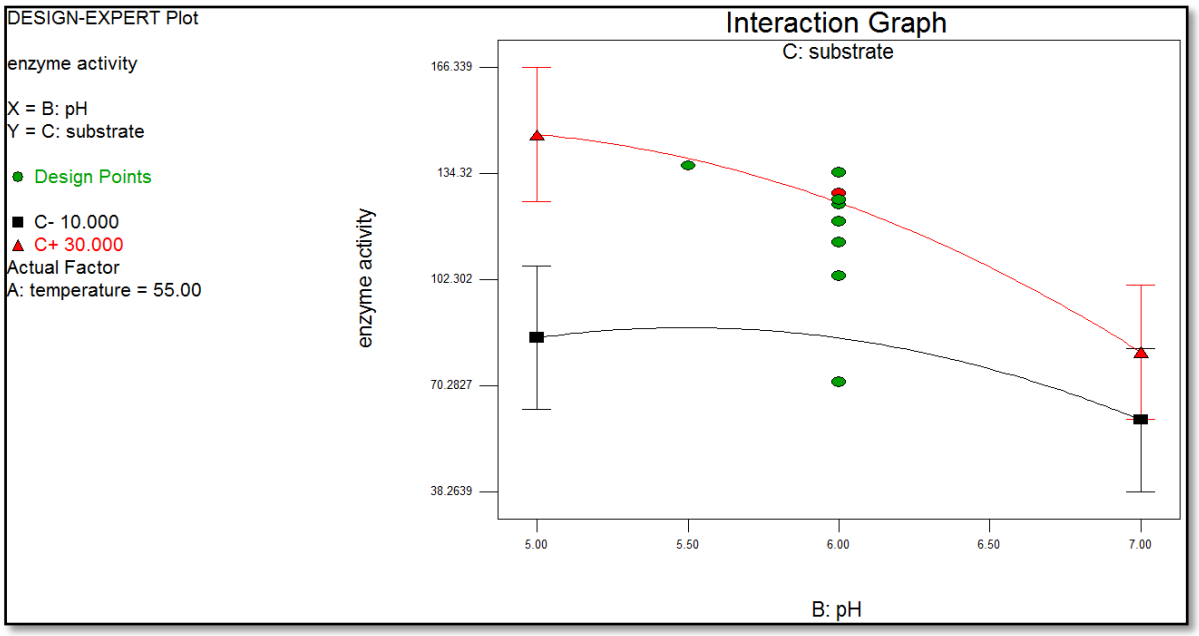


Figure 4.15: Interaction graph of purified enzyme activity from the model equation: effect of pH and substrate concentration (g/L)

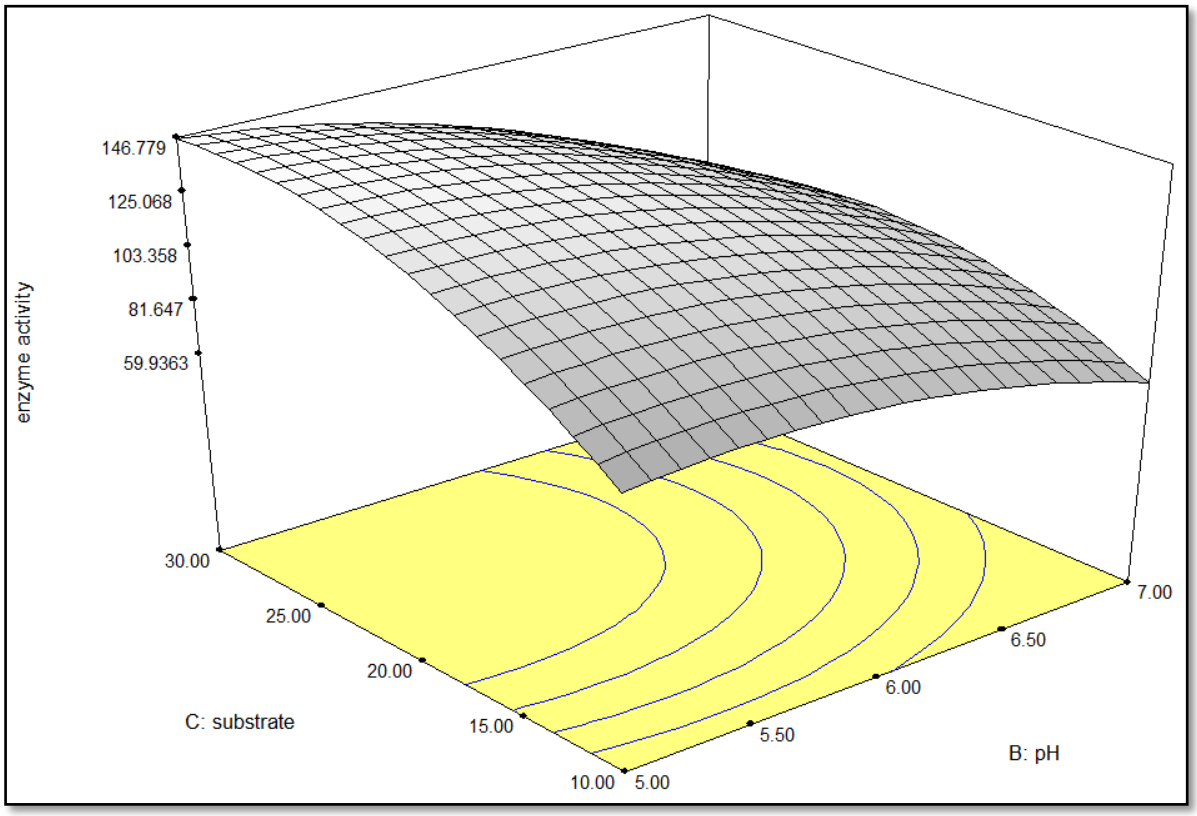


Figure 4.16: Three dimensional (3D) graph of purified enzyme activity from the model equation: effect of pH and substrate concentration (pH 5.0 to 7.0 and substrate concentration 10 – 30 g/L)

4.3 VALIDATION OF EMPIRICAL MODEL ADEQUACY

Adequacy of the developed empirical model needs to be verified or validated in order to confirm the prediction accuracy, which is generated by the regression equation in predicting the enzyme activity at any particular reaction pH, temperature and substrate concentration within the range of level defined previously. Experimental rechecking was performed using conditions that were previously used and combined with the additional experiments which have not been tried before but was within the limits tested. The obtained actual values and its associated predicted values from the selected experiments were compared for further residual and percentage error analysis. The percentage error between actual and predicted value of the response (enzyme activity) over a selected range of operating levels are calculated based on Equation 4.3 and 4.4.

Table 4.8: Results of operating conditions with experimental design in validation experiment

No	Temperature (°C)	pH	Substrate Concentration (g/L)	Predicted	Actual	Residual	% Error
1	60.00	5.00	26.49	135.81	129.41	-6.40	4.95
2	60.00	5.00	26.23	135.78	128.50	-7.27	5.67
3	60.00	5.01	26.38	135.67	129.03	-6.64	5.15
4	60.00	5.03	26.48	135.41	127.39	-8.02	6.30
5	53.38	5.00	27.82	133.76	134.07	0.31	0.23

$$Residual = (Actual\ value - Predicted\ value) \tag{4.3}$$

$$\%\ Error = \frac{Residual}{Actual\ \ value}\ x\ 100\% \tag{4.4}$$

Table 4.8 shows the percentage error is ranging from 0.23% to 6.30% for FTase activity. Thus implied that the empirical model developed was considerably accurate for responding term which is enzyme activity as percentage error between the actual and predicted values were well within the value of 10%, suggesting that the model adequacy is reasonably within the 90% of the prediction interval. This means further analysis with regards to ideal operational process for optimal FTase activity would be based on this developed model.

4.6 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL (SDS PAGE)

After partially purifying the sample of FTase, SDS-PAGE experiment was used to identify fraction that contain the targeted protein by separating the proteins according to their molecular weight. This was done first through gel electrophoresis of the collected sample. These were loaded alongside known standards, and then allowed to run along the gel in order to separate based on molecular weight. But, there are some problem occurred during this study which SDS-PAGE experiment cannot be completed. The problem such as the mobility of protein cannot be detected thus molecular weight of FTase cannot be determined. It is maybe because of the buffering gel and stacking gel is mixed. The second problem is the gel of SDS-PAGE is too fragile which it is easy to break.

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The main objective of this study which is to purify the FTase from *Aspergillus niger* is achieved. The kinetic study shows the enzymes FTase were neutral enzyme and need to be reacted in neutral condition which is 5.0 to 6.0. The activity of enzyme also studied and based on the result, purified enzyme has the highest enzyme activity compared to crude enzyme activity. The optimum condition for enzyme is at pH 5.5, temperature at 55 °C and substrate concentration at 25 g/L. The maximum activity of purified enzyme is at 132.169 U/mL while the maximum activity of crude enzyme is observed at 127.705 U/mL. In this phase, the response surface methodology (RSM) was applied to get the optimum value of the enzyme activity based on the three variables: substrate concentration, temperature and pH and after the optimization process done, the maximum value of the enzyme activity was achieved.

5.2 RECOMMENDATION

A number of recommendations are being proposed in order to optimize the enzyme activity to produce high yield of FOS are being listed as below:

1) Determination of other parameter.

   Parameters that have been studied in this experiment are effect of pH, substrate concentration and temperature towards the enzyme activity. For the future study, additional parameter can be added such as cultivation time, inhibition of other material towards enzyme activity and agitation speed in order to understand the enzyme characteristic or to find other way to improve the enzyme activity.

2) Using other microbial organism to produce enzyme

Aspergillus niger has been used in this study to produce FTase. There are many more microbial organisms such as *Penicillium simplicissimum*, *Aureobasidium pullulan* and *Arthrobacter* sp. can produce FTase. Different microbial organism produce different yield of FTase and by using different microbial organism, we can determine the microorganism that produce high yield of enzyme.

3) SDS-PAGE

Based on the SDS PAGE experiment, there is an inconsistency problem occurred to the process run. In the future studies, TEMED and ammonium persulfate should be leaved into gel mixture when the gel sandwich is correct assemble and there are no leaking occurs. In this study, the proposed staining solution is by using Coomasie brilliant blue staining. For the future study, the staining solution can be replaced by silver staining which is can give more accurate results. To deal with SDS-PAGE gel, precaution steps need to be taken so that the gel will not easily break by gently handling on the gel.

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APPENDIX A1

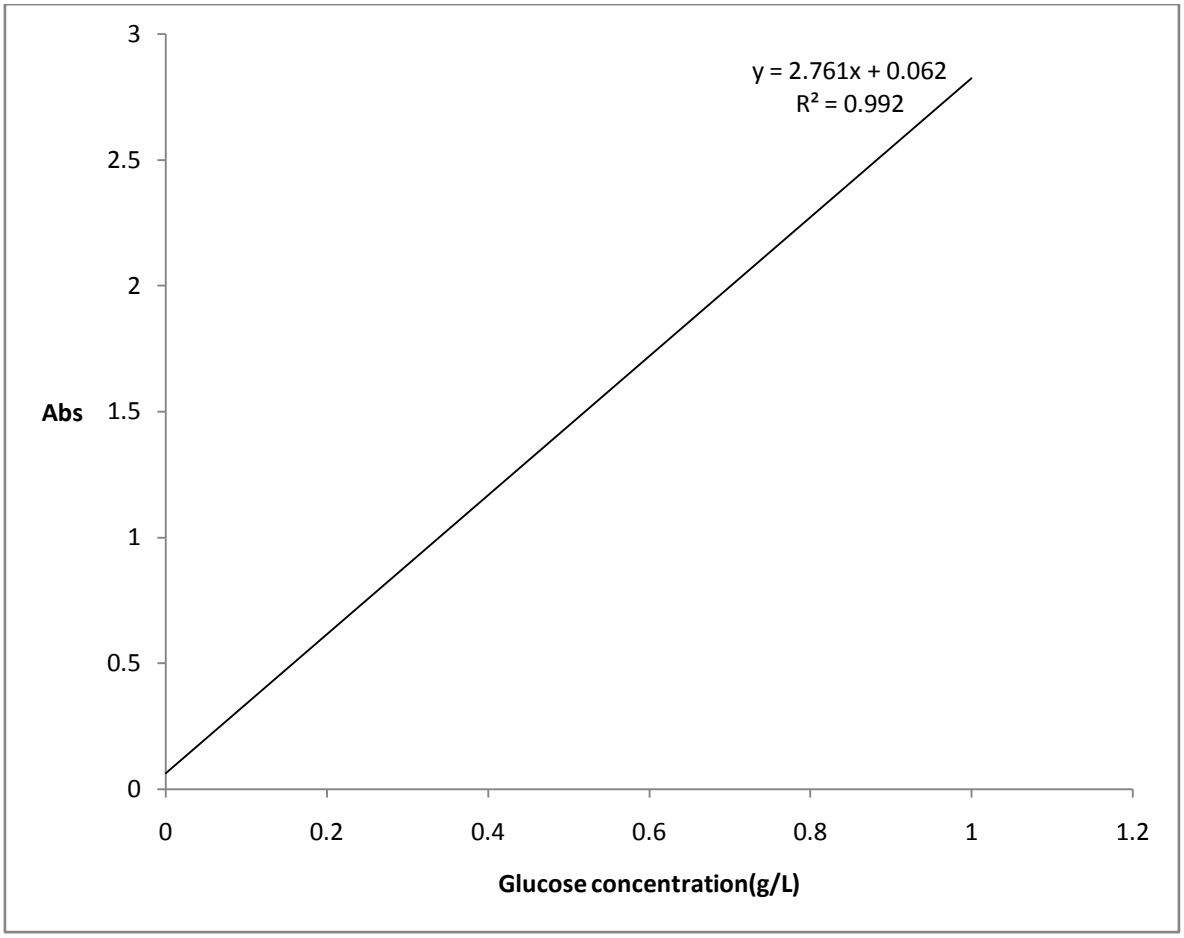

Figure A1: Standard calibration curve of glucose

APPENDIX A2

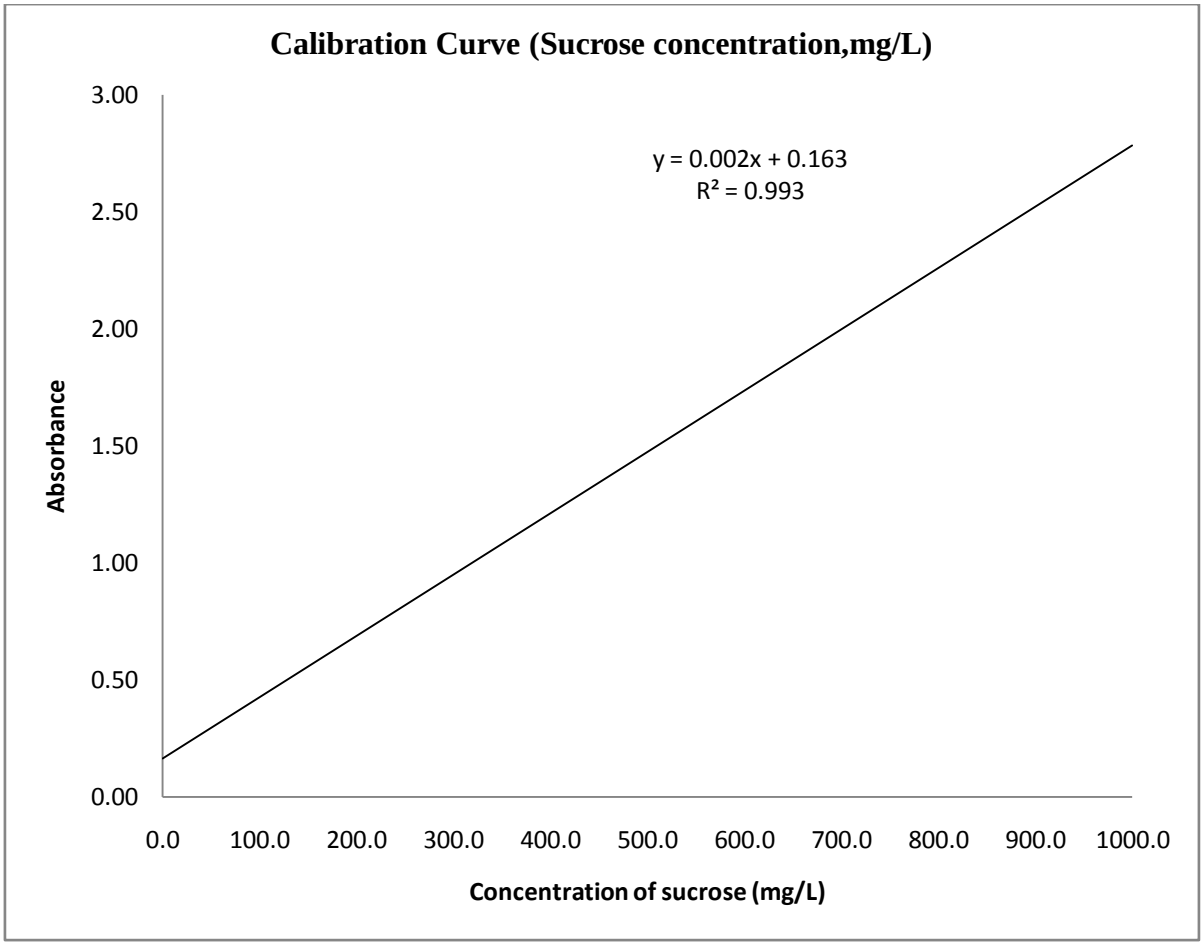

Figure A2: Standard calibration curve of sucrose