CHARACTERIZATION AND THERMOSTABILITY STUDY OF INVERTASE BY *ASPERGILLUS NIGER* IN SUBMERGED CULTURE

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SUPERVISOR'S DECLARATION

"I hereby declare that I have checked this thesis and in my opinion this thesis has fulfilled the qualities and requirements in terms of scope and quality for the award of Degree of Bachelor of Chemical Engineering (Biotechnology)"

Signature : .....

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Thesis submitted in fulfillment of the requirements for the award of Degree of Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical Engineering and Natural Resources

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except as cited in references. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature : .....

Name : NURUL IZZAH BINTI AHMAD

Date : .....

Special dedicated to my family, my supervisor, my friends, my fellow colleague,

and to all faculty members

For all your care, support and believe in me.

Sincerely;

Nurul Izzah binti Ahmad

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In the name of Allah, the Most Gracious and Most Merciful.

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ABSTRACT

Invertase is a commercially important enzyme used for hydrolysis of sucrose. The hydrolysis of sucrose yields a mixture of glucose and fructose, or famous as name invert syrup, this enzyme was widely used in food and beverage industries. Objectives of this research are to study the pH and thermostability of invertase by *Aspergillus niger*, to study the enzyme kinetics of invertase and to study the effect of sucrose concentration during incubation towards invertase activity. *A. niger* produced high levels of invertase under culture conditions (potato dextrose agar) on fourth day of incubation at an optimum temperature 30^{O}C, inoculum at 30^{O}C at 250 rpm and using sucrose as a substrate by submerged fermentation (SmF) also with same culture conditions. Separation between enzyme and other cell was done by centrifugation and continued with study the pH and thermostability of enzyme by terminated by heating. Thermostability of enzyme were investigated by determining the enzymatic reaction subjected to 40^{O}C, 45^{O}C, 50^{O}C, 55^{O}C and 60^{O}C. Michaelis-Menten parameters (V_{max} and K_m) were determined for four different sucrose concentration which are 0.2, 0.4, 0.6, 0.8 g/L. Result suggested that optimum pH is 5.0 and temperature is 55^{O}C. While both V_{max} and K_m would increase at higher temperatures because temperature will alter the shape of enzyme by changing its ionic form at active site.

ABSTRAK

Invertase adalah enzim komersial penting yang digunakan untuk hidrolisis sukrosa. Proses hidrolisis sukrosa menghasilkan campuran glukosa dan fruktosa, atau terkenal sebagai nama terbalikkan sirap, enzim ini telah digunakan secara meluas dalam industri makanan dan minuman. Objektif kajian ini adalah untuk mengkaji kestabilan pH dan suhu invertase, untuk mengkaji enzim kinetik invertase dan untuk mengkaji kesan kepekatan sukrosa semasa inkubasi terhadap aktiviti invertase. *Aspergillus niger* menghasilkan invertase pada tahap tinggi bawah keadaan kultur (kentang agar dekstrosa) pada hari keempat pada suhu optima 30^{o}C, inokulum 30^{o}C pada 250 rotasi per minit dan menggunakan sukrosa sebagai substrat oleh kultur tenggelam juga dengan keadaan kultur yang sama. Pengasingan antara enzim dan sel lain telah dilakukan melalui proses pemusingan dan teruskan dengan kajian kestabilan pH dan suhu enzim diakhiri dengan pemanasan. Kestabilan suhu enzim telah dinilai dengan menentukan tindak balas enzim terhadap 40^{O}C, 45^{O}C, 50^{O}C, 55^{O}C dan 60^{O}C. Michaelis-Menten parameter (Vmax dan Km) telah ditentukan oleh empat kepekatan sukrosa 0.2 g/L, 0.4 g/L, 0.6 g/L dan 0.8 g/L. Keputusan mencadangkan bahawa pH optima ialah 5.0 dan suhu optima adalah 55^{O}C. Walaupun kedua-dua Vmax dan Km akan meningkat pada suhu yang lebih tinggi kerana suhu akan mengubah bentuk enzim dengan menukar bentuk ionik di tapak aktif.

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LIST OF SYMBOL / ABBREVIATIONS

HFS	High Fructose Syrup
K_m	Maximum Reaction Rate or substrate concentration when the enzyme has half-maximal velocity
V_{max}	Maximum Velocity
GRAS	Generally Recognized as Safe
S	Substrate
E	Enzyme
ES	Intermediate complex
MARDI	Malaysian Agricultured Research and Development Institute
PDA	Potato Dextrose Agar
HPLC	High-Performance Liquid Chromatography
$(NH_4)_2SO_4$	Ammonium Sulphate
$FeSO_4$	Iron Sulphate
$MgSO_4$-$7H_2O$	Magnesium Sulfate Heptahydrate
rpm	Revolutions per minute
ppm	Parts per million
SmF	Submerged Fermentation
SSF	Solid State Fermentation
IU/mL	Concentration of one international unit per one millilitre of system volume

CHAPTER 1

INTRODUCTION

1.1 Background of Proposed Study

Enzymes are proteins that produced by all living cells as catalysts for specific chemical reactions. In enzymatic reactions, the molecule at beginning is called substrates, and then they convert into different molecules called products. Almost all biological cells need enzymes to increase the rate of chemical reactions in order to occur at rates sufficient for life. Enzymes are very important part in some food processes, such as making of cheese, bread, wine and beer, for thousands of years (Dewdney, 1973). Invertase (invert sugar) is one of the most widely used enzymes in confectionery industry for preparation of jams and candies (Klein *et al*. 1989). Invertase or β-fructofuranoside catalyzes the cleavage of sucrose to glucose and fructose. Invertase usually used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS).

Invertase must be one of the best known of all enzyme systems because of its historical importance, but only recently have its properties been subjected to rigorous examination. Recently, many research studied about enzyme kinetics on invertase activity in dilute solution (Uma *et al*., 2012). More recently there has been an increment of interest in its catalytic properties at higher concentrations of substrate and products because of commercial applications in the production of liquid, free sugar solutions. This study has good potential for food biotechnological applications in increasing biotechnological importance of thermostable invertase.

1.2 Problem Statement

Use of enzymes as catalysts is limited by their stability on storage. Their stability will decreases due to changes in pH and temperature as a result of friction, osmotic pressure imposed by the environments of their use and a cumulative effect of all these factors as a function of duration of their use (Kotwal and Shankar, 2009). As temperature rises, molecules have more kinetic energy. And this will increase collision between them and the rate increase. While when pH changes, intermolecular bond break and thus changing its shape and the enzyme become effectiveness.

Enzymatic reactions may be contributed to a significant extent to these transformations. Enzymatic reactions may be desirable in some cases but are often disturb. Control of such reactions is necessary for technological improvement (Drapon, 1985) as well as preservation of quality and shelf life of foods. In industrial processes, finding a working temperature that gives high enzyme activity with good

stability represents a compromise between lower process costs and higher productivities. The selection of suitable temperature that may improve enzyme stability without considerable loss of activity is one of the main purposes of this study.

The kinetic parameters describing the catalytic mechanism of the enzyme are independent of substrate concentration. Variations in pH of medium result in changes of ionic form of the active site thus alter the shape of enzyme and lastly affect the maximum reaction rate, K_m. If an enzyme has a small value of K_m, and it achieves its maximum catalytic efficiency at low substrate concentrations. However, the value of K_m for an enzyme depends on the particular substrate. It also depends on the pH and temperature of the solution at which the reaction is carried out. Lower K_m values are generally associated with lower V_{max} values (Somero, 1978).

1.3 Objectives

1.3.1 To study the pH and thermostability of invertase by *Aspergillus niger*.

1.3.2 To study the enzyme kinetics of invertase.

1.3.3 To study the effect of sucrose concentration during incubation towards invertase activity.

1.4 Scope of Study

This study will be carried out in submerged fermentation using 500 mL shake flask culture. For stability study, the effect of temperature ranged for 40^{O}C, 45^{O}C, 50^{O}C, 55^{O}C, 55^{O}C and 60^{O}C and pH ranged from 4.0, 5.0, 6.0, 7.0 and 8.0 will be investigated. For enzyme kinetic determination (K_m and V_{max}), the initial rate concentration will be estimated at substrate concentration at range 0.2 g/L, 0.4 g/L, 0.6 g/L and 0.8 g/L. Sucrose concentration also was studied on invertase activity at 0.2 g/L, 0/4 g/L, 0.6 g/L and 0.8 g/L.

1.5 Rational and Significance

From this study, we can know most stable condition of invertase and from that we should be more alert and produce high quality food. Invertase is one of the essential nature enzymes uses to help us to digest sugars. It is commonly found in bee pollen and yeast sources, other than that, invertase also plays a key role not only in digestive processes, but important in human disease prevention, physical rejuvenation and anti-ageing processes. Theoretically, as we age, we have less access to this natural enzyme which resulting in a reduced ability to extract the vital nutrients from the food that we eat. It can also slow down our digestive processes, as sugars and starches are such a big part of most people diets. While some forms of sugar and carbohydrates are good for the body, they cannot be absorbed or digested well without the help of the invertase enzyme (Edward, 2011).

Other than that, study of stability of enzymes is an important aspect to consider in biotechnological processes as this can provide information on the structure of enzymes and facilititate an economical design of continuous processes in bioreactors (Jurado *et al*., 2004). The enzyme has excellent activity at lower temperatures, but its reaction times had to be extended. To compensate for the extended reaction times, more enzymes may be used. For ideal results, invertase can be used at temperatures up to 60°C without loss of activity. But then if temperature above it, enzyme activity begins to decrease, more enzymes may be used to compensate for the loss of activity. It may be necessary to optimize applications requiring higher temperatures depending on its actual temperatures. Invertase may be used over an extended pH range with an optimum pH at 4.5.

Prolonged exposure to extreme heat and humidity can denature enzymes more rapidly and result in loss of activity. Enzymes also lose activity due to oxidation or hydrolysis. These phenomena are inherent in the enzyme, and there is little that can be done. Because of these inherent characteristics, enzymes need to be carefully stored, handled, formulated and encapsulated. It is recommended that enzymes be stored in a cool and dry environment. In addition, other conditions such as the high acidity of the gut where some enzymes may be sensitive can be overcome with enteric coatings or microencapsulated enzymes.

Anderson (2011) also stated that when it comes to enzyme formulation, stability is key point. For better enzyme, test for stability and activity, and then test again if excipients have been added. And while an enzyme's sensitivity to its environment may mean taking extra care in storage and processing, it is that same highly responsive nature that makes an enzyme finely attuned to the digestive

processes as it supports if it is properly formulated. For instance, a digestive enzyme can be prematurely activated or destroyed in the acid of the stomach, rendering it useless for nutrient absorption.

CHAPTER 2

LITERATURE REVIEW

2.1 Invertase

Invertase or official name beta-fructofuranosidase is a commercially important enzyme used for the hydrolysis of sucrose. This hydrolysis of sucrose yields an equimolar mixture of glucose and fructose, known as invert sugar. Invert sugar is sometimes referred to as artificial honey since its composition and properties are nearly same. Figure 2.1 shows how sucrose hydrolysis by invert sugar. Enzymatic invert sugar is safe as it also an absolute health-friendly sweetener. So, the enzyme invertase is employed for the hydrolysis of sucrose and fructose. Nowadays, fructose is a sugar which can be consumed by diabetic patients. This because its metabolism which is insulin independent. Also, invert sugar is 28 to 30% sweeter than table sugar so the amount of sugar required for particularly degree of sweetness is also reduced. Finally, the enzymatic invert sugar does not involve the

use of any chemicals or acids unlike conventional acid-hydrolyzed invert syrup. Hence, it is completely healthy sweetener (Willkommen, 2007).

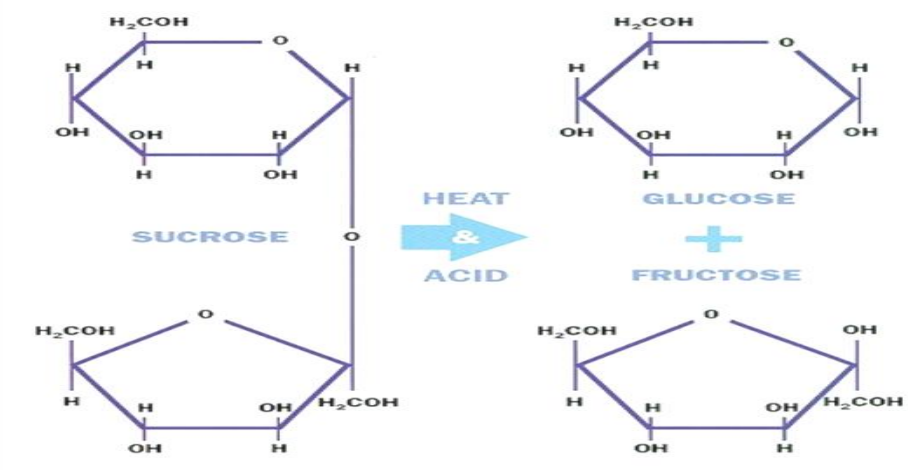

Figure 2.1: Hydrolysis of Sucrose Producing Glucose and Fructose

Traditionally, invertase usually produced on site by autolysing yeast cells and is sold either as a clear liquid or as a powder that can be dissolved in water. Its name refers to its ability to change the direction of optical rotation of sucrose solution as a result of hydrolysis to glucose and fructose (Whitaker, 1972). According to Verma and Dubey (2001) invertase is a group of ubiquitous enzymes with different pH optimum and subcellular localization. Invert sugar is frequently used in commercial baking and candy recipes because it keeps baked goods moist for longer periods of time. The invertase itself should be stored in the refrigerator for longevity. Optimum conditions for invertase are pH 3.0 to 6.0 and temperature 40^{O}C to 80^{O}C.

2.2 Application of Invertase

Invertase is widely used in food (confectionery) and beverage industries where fructose is preferred over sucrose because it is sweeter and does not crystallize easily. However, the use of invertase is limited because another enzyme, glucose isomerase can be used to convert glucose to fructose more inexpensively. For health and taste reasons, its use in food industry requires that invertase be highly purified. This enzyme is also used for the manufacture of artificial honey, plasticizing agents that commonly used in cosmetics, pharmaceutical industries and paper industries as well as enzyme electrodes for the detection of sucrose, high solubility, humid nature and high osmotic pressure. Invertase is used for production of inverted sugar, the valuable commercial product for the food industry in countries where the main sources of sugar is beet or cane. (Research Article, retrieved from http://scialert.net).

Klein *et al.* (1989) state that invertase catalyzes the cleavage of sucrose to glucose and fructose. Today, invertase is one of the most widely used enzymes in food industry, especially in preparation of jams and candies. Other than that, this invert sugar can be used in biscuits as caramelisation, enhanced flavour and better texture, bread, cake and pastries as better crust colour and softer crumb and faster yeast activation, fruit processing for better shelf life and provides better taste profile, and squashes, lemonades and instant energy drinks because glucose and fructose provide instant energy and better taste (retrieved from http://www.ensymm.com/). Invertase also used in pharmaceutical industry as digestive aid tablets or ready to use for cough syrups and glucose and fructose based intravenous fluids, powder milk for

infants' food, as calf feed preparation, assimilation of alcohol in fortified wines and manufactured inverted sugars as food for honeybees (Uma *et al*., 2012).

2.3 *Aspergillus niger*

Aspergillus niger is a widely distributed filamentous fungus (Bos et al., 1988). Bakri et al., 2003 stated and proved that filamentous fungi are preferred for commercial enzyme production, because the level of the enzyme of these cultures is higher than those obtained from yeast and bacteria. *A. niger* has been single largest fungal source of enzymes (Subramaniyam and Vimala, 2012). *A. niger* produce colonies that are mainly composed of white or yellow that was covered by dark fungal spores. This fungus often found indoors and grows as black colonies called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and basically known as a contaminant of food. It is ubiquitous in soil and commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys chartarum*, toxic black mold species of which can infest water-damaged dwellings and cause severe symptoms in people. Black mold is ever-present in soil and decaying plant such as compost piles. Its spores are very common in air. *A niger* also is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and in severe cases, damage to the ear canal and tympanic membrane. *A. niger* invades the outer ear canal which can cause damage to the skin it came in contact with (May, 1997).

A. niger is a haploid filamentous fungi and is a very essential microorganism in the field of microbiology. In addition to producing extracellular enzymes and citric acid, *A. niger* is always used for waste management and biotransformation. The fungi is most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Schuster *et al*. 2002). Genome sequencing of *A. niger* is important because of its involvement in producing citric acid as well as other industrial enzymes, such as amylases, proteases, and lipases. The use of these enzymes is essential because of its importance for transformation to food enzymes. Other properties of this species include pathogens that cause the spoilage of food and production of secondary metabolites, such as aflatoxin, that are toxic. Understanding this economic importance as well as the effects it makes on the environment makes the genome sequencing of *A. niger* essential to biological applications (Takahashi *et al.*, 1991; May., 1997).

Biologically enzymes commonly isolated from any living organism such as plants, animals and microorganism. But now, microbe is preferred as sources of enzymes because of less harmful materials than plant and animal tissues. Moreover, according to Uma *et al.,* 2012, majority of enzymes used in industrial or in biotechnological applications are derived from fungi and bacteria. *A. niger* has been a very important microbe used in the field of biotechnology. Many of the industrial enzymes produced by *A. niger* such as citric acid, amylases, lipases, cellulases, xylanases and proteases, are considered GRAS (generally recognized as safe) by the United States Food and Drug Administration and is excused from the Federal Food, Drug, and Cosmetic Act food additive tolerance requirements. However, it is still important to treat this fungus very carefully to avoid formation of spore dust which can cause illness (Schuster *et al*., 2002).

2.4 Effect of pH on Invertase Production

Efficient thermostable invertase was active in acidic pH. The maximum invertase production was detected by using sucrose as carbon source. At higher pH values, enzyme was higher in the medium supplemented with low sucrose concentration. It was observed that the more of fungi decreased, the greater the enzyme activity observed. It is suggested that invertase may precipitate in autolysis of fungi and could prove to be a potential source of industrial application and exploitation (Vaishali and Vrinda, 2009).

Table 2.4: Effect of pH on Invertase Production

pH	Optimum Activity of Invertase	References
4.6 to 6.5	pH 5.5, 0.7 IU/mL	Boddy *et al.*, 1992
3.0 to 8.0	pH 5.0, 23 IU/mL	Uma, 2010
2.0 to 8.0	pH 5.0	María and María, 1995
3.5 to 5.5	pH 4.5	Nam, 2009
2.0 to 11	pH 4.4	L'Hocine *et al.*, 2000
4.0 to 10	pH 5.0	Kaur and Sharma, 2005
4.0 to 9.0	pH 4.5	Rubio *et al*., 1997
4.0 to 9.0	pH 5.0	Sirisansaneeyakul *et al*., 2000

2.5 Effect of Temperature on Invertase Production

Invertase activity also effected by temperature, as the temperature increases, the rate of reaction also increase, but when it reach at certain temperature the rate of reaction drops drastically which explained by the nature of the enzymes which are proteins. All proteins with tertiary structure, like invertase, denature when exposed to high temperatures. Denatured proteins do not react as much as normal proteins, therefore lessening the reaction rate of the system (Lusung and Maningat, n.d.)

Table 2.5: Effect of Temperature on Invertase Production

Temperature	Optimum Activity of Invertase	References
20^{o}C to 60^{o}C	50^{O}C, 40 IU/mL	Uma *et al.*, 2010
20^{o}C to 70^{o}C	50^{O}C, 3.2 IU/mL	Boddy *et al.*, 1993
25^{o}C to 75^{o}C	60^{O}C	María and María, 1995
30^{o}C to 70^{o}C	55^{O}C,	L'Hocine *et al.*, 2000
60^{o}C to75°C	30°C	Rajoka and Yasmeen, 2005
20^{o}C to 90^{o}C	60^{o}C	Lusung and Maningat, n.d.
50^{o}C to 70^{o}C	45^{o}C	Kaur and Sharma, 2004

2.6 Michaelis-Menten Kinetics

From McKee and McKee (1946), one of the most useful models in the systematic investigation of enzymes rates was proposed by Leonor Michaelis and Maud Menten in 1913. The concept of enzyme-substrate complex, first enunciated by Victor Henri in 1903, is central to Michaelis-Menten kinetics. When the substrate S binds in active site of an enzyme E, an intermediate complex (ES) is formed. During transition state, the substrate is converted into product. Then the product dissociates from the enzyme. This process can summarize as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

where k_1 = rate constant for ES formation

k_{-1}= rate constant for ES dissociation

k_2 = rate constant for product formation and release from active site

The dependence of an initial rate of reaction upon the concentration of a substrate S that is present in large excess over the concentration of an enzyme or other catalyst (or reagent) E with the appearance of saturation behavior following the Michaelis–Menten equation:

$$v = \frac{V_{\max}[S]}{[S] + K_m} \qquad (2)$$

where v is the observed initial rate

V_{max} is its maximum velocity

K_m the substrate concentration when the enzyme has half-maximal velocity

The Michaelis constant K_m is defined as the substrate concentration at half the maximum velocity V_{max}. Michaelis developed the following expression for the reaction velocity in terms of this constant and the substrate concentration. Michaelis constants have been determined for many of the commonly used enzymes. The size of K_m can tells us several things about a particular enzyme.

1. A small Km indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.

2. A large Km indicates the need for high substrate concentrations to achieve maximum reaction velocity.

3. The substrate with the lowest Km upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes. (Retrieved from http://www.worthington-biochem.com/introbiochem /substrateconc.html).

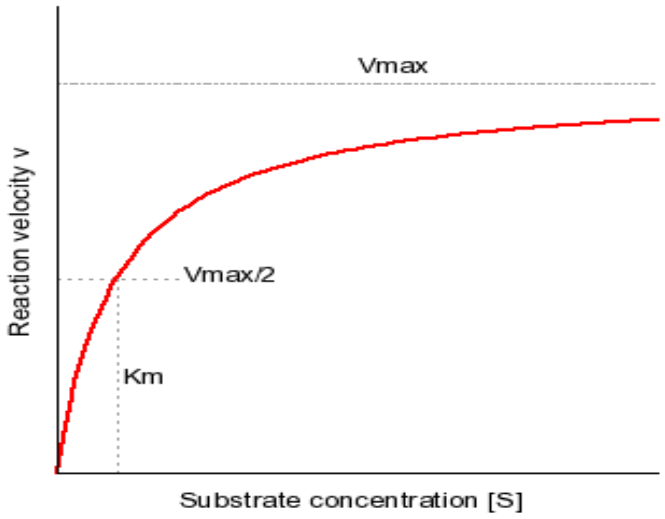

Figure 2.6.1: Michaelis-Menten Model

Source: Shuler and Kargi 2002

Lineweaver-Burk analysis is one method of linearizing substrate-velocity data as to determine the kinetic constants K_m and V_{max}. One creates a secondary, reciprocal plot: 1/velocity vs. 1/[substrate]. When catalytic activity follows Michaelis-Menten kinetics over the range of substrate concentrations tested, the Lineweaver-Burk plot is a straight line with y intercept = $1/V_{max}$, x intercept = $-1/K_m$ and slope = K_m/V_{max} (Shuler and Kargi, 2002).

$$\frac{1}{V} = \left[\frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}} \right] \tag{3}$$

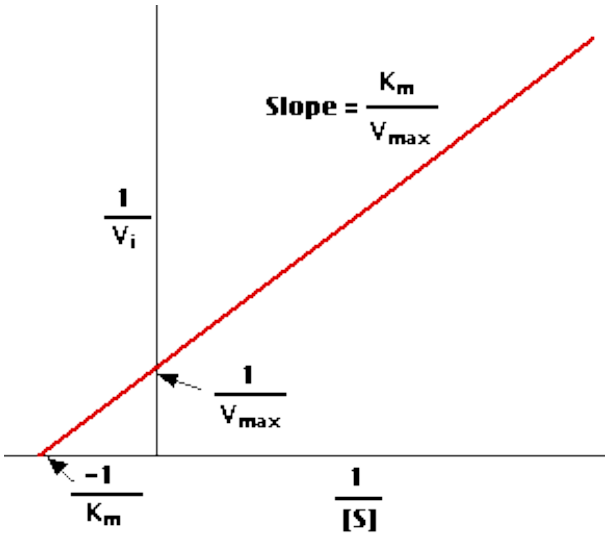

Figure 2.6.2: Lineweaver-Burk Analysis

Source: Shuler and Kargi 2002

2.7 Fermentation Methods for Invertase Production

Fermentation is a technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi (Subramaniyam and Vimala, 2012). Fermentation has been widely used in enzyme production that is highly beneficial to individuals or industries. Over the years, fermentation techniques have gained immense importance due to their economical and environmental advantages. Early techniques have been further modified and refined to maximize productivity. Fermentation has been classified into Solid State Fermentation (SSF) and Submerged Fermentation (SmF) mainly based on the type of substrate used during fermentation. SSF utilizes solid substrates, like bran, bagasse, and paper pulp. In this technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. SmF utilizes free flowing liquid substrates, such as molasses, broths and liquid media. The substrates are utilized quite rapidly and need to be constantly replaced with nutrients. This technique is best suited for microorganisms such as bacteria that require high moisture content. So for this study, SmF was choosing as better technique as sucrose was used as substrate. Table 2.7 below shows advantages and disadvantages of SmF.

Table 2.7: Advantages and Disadvantages of SmF (Mitchell and Losane, 1992; retrieved from http://www.scribd.com/doc/48369336/Submerged-fermentation)

Advantages	**Disadvantages**
Measure of process parameters is easier.	High cost due to expensive media.
Bacteria and fungi cells are distributed throughout the medium.	Risk of contamination.
High water content.	High quantity of liquid waste produced.
Culture medium always free flowing	
Whole system is always under aseptic condition.	

According to Uma *et al*., 2012, invertase production was found to be higher in SmF than in SSF. The strain was grown in shaken flasks filled with nutrient and carbon source. For SSF, it's using polyurethane foam (PUF) and autoclaved separately from nutrient and carbon source. But *A. niger* did not grow on PUF and lack of either carbon or nutrients. By refer to the result, although strain grew faster in SmF compared to SSF, PUF in SSF seen not reasonable as its medium culture is not as limited by oxygen transfer as in case of SmF culture (Marsh *et al*., 1998).

CHAPTER 3

METHODOLOGY

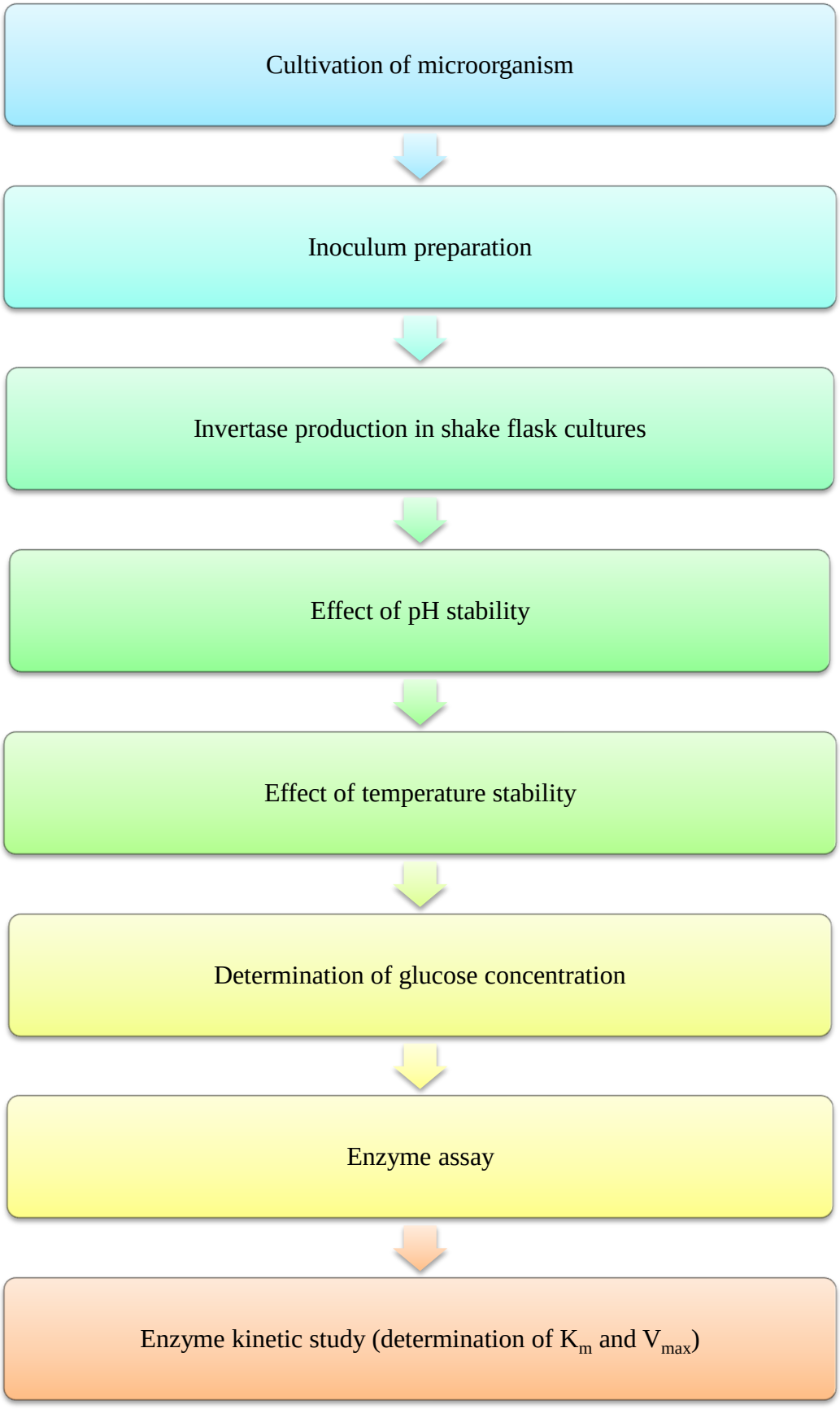

Figure 3.1: Flowchart of Overall Experimental Activities Involved in This Study

Cultivation of Microorganism

Aspergillus niger was procured from Malaysian Agricultured Research and Development Institute (MARDI). The culture was grown on potato dextrose agar (PDA) at $30^{O}C$ for 4 days. The stock culture were maintained on PDA at $4^{O}C$ and subcultured every 14 days.

Figure 3.2: *Aspergillus niger* on PDA

Inoculum Preparation

The *Aspergillus niger* spores from the PDA slant were used for inoculum preparation. The composition of the medium used for the inoculum comprised of 30% sucrose and 1 % yeast extract, respectively. 250 mL of inoculums broth with *A. niger* strain was incubated at 30°C, 250 rpm for 24 hours in incubation shaker.

Invertase Production of Invertase in Shake Flask Cultures

The medium used for enzyme production under submerged fermentation comprised of 45g/L of ammonium sulphate $(NH_4)_2SO_4$, 0.1 g/L of iron sulphate $FeSO_4$, 7 g/L of $MgSO_4$-$7H_2O$, 1% of yeast extract and 30 g/L of sucrose. Fermentation medium was adjusted to pH 5.5 with 0.1 M Sodium Hydroxide NaOH and 0.1 M Hydrochloric

Acid, HCl. Fermentation was carried out at 30^{O}C and pH 5.5 for 40 hours in 500 mL shaken flasks containing 235 mL of fermentation medium and 15 mL of inoculum at agitation rate 250 rpm. This stock solution was prepared in 5 flasks. The stock solutions were centrifuged at 12 000 rpm for 20 minutes at 4^{O}C.The supernatant was used as crude enzyme.

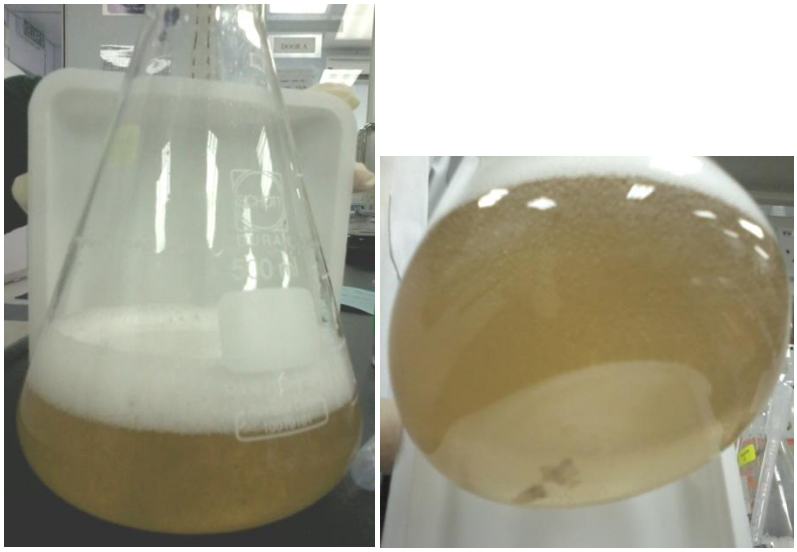

Figure 3.3: Cell after Inoculum and Fermentation

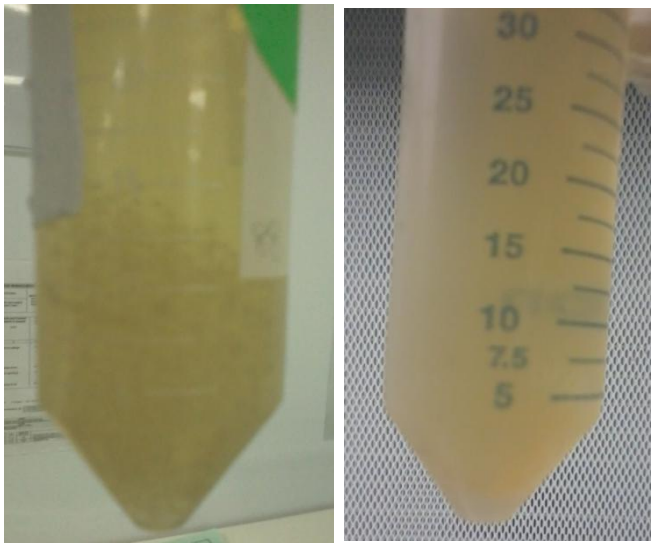

Figure 3.4: Enzyme Solution Before and After Centrifuge

Effect of pH Stabilization

The ranged of pH tested, from 4.0 to 8.0, was obtained by adding to the reaction mixture of various pH buffer (Acetate buffer for pH 4 and pH 5), Phosphate buffer for pH 6, pH 7 and pH 8) and 30% sucrose.

Effect of Temperature Stabilization

The effect of temperature on invertase stability was monitored by assaying the enzyme at 40, 45, 50, 55 and 60°C in water bath for 1 hour. The reaction mixture was terminated in at 100°C for 15 minutes and the invertase activity was then determined.

Standard Curve Glucose

Glucose concentration was determined from its chromatographic peak area measured using a pre-determined calibration curve (0.0 g/L,0.2 g/L, 0.4 g/L, 0.8 g/L and 1.0 g/L).

Enzyme Assay

The enzymatic activity was determined in reaction mixture consists of 30% sucrose (1 mL) as the substrate, 0.2 M acetate buffer at the required pH 4.5 (1 mL) and enzyme solution (0.5 mL). The enzyme reactions was carried out at pH 4.0, 5.0, 6.0, 7.0 and 8.0 and temperature 40^{O}C, 45^{O}C, 50^{O}C, 55^{O}C and 60^{O}C for 2 hours and terminate by heating at 100^{O}C for 15 minutes. The glucose concentration in terminated stock solution was estimate by using high-performance liquid chromatography (HPLC). The HPLC was performed as follows: sugar column (Luna 5u NH2 100A), flowrate 1 mL/min and mobile phase with ratio acetonitrile:

deionised water 75:25. The column temperature was maintained at 40^{O}C. Column eluent was detected with refractive index detector in HPLC.

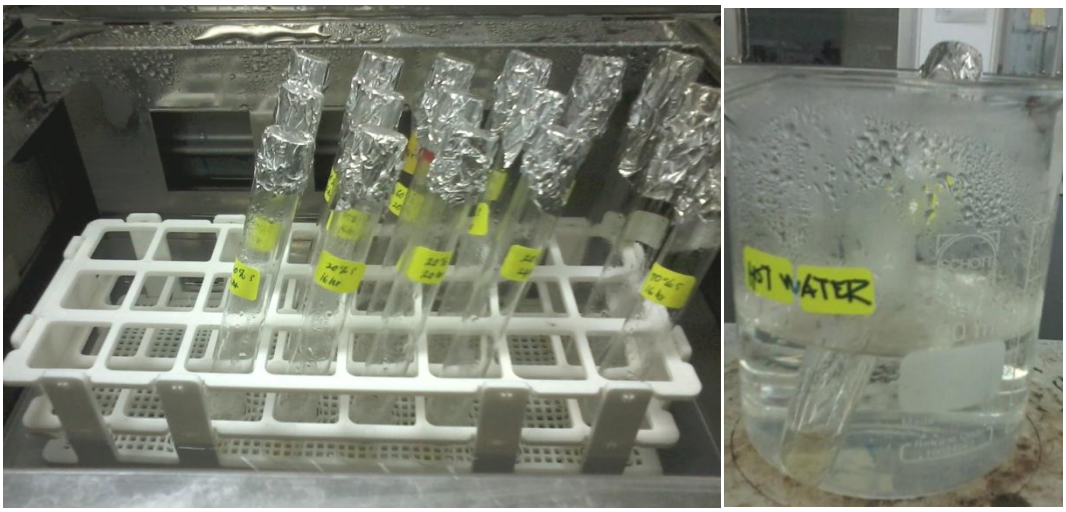

Figure 3.5: Enzyme Assay of Invertase

Enzyme Kinetic Study (Determination of K_m and V_{max})

The Michaelis–Menten kinetics was established from a study of effect of substrate concentration on the reaction rate. The reaction was conducted with various sucrose concentrations (20 g/L, 40 g/L, 60g/L and 80 g/L) and the rate of reaction at each concentration was determined. The Michaelis constant, K_m and V_{max} were obtained from the Lineweaver-Burk plot.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Effect of pH on Invertase Stability

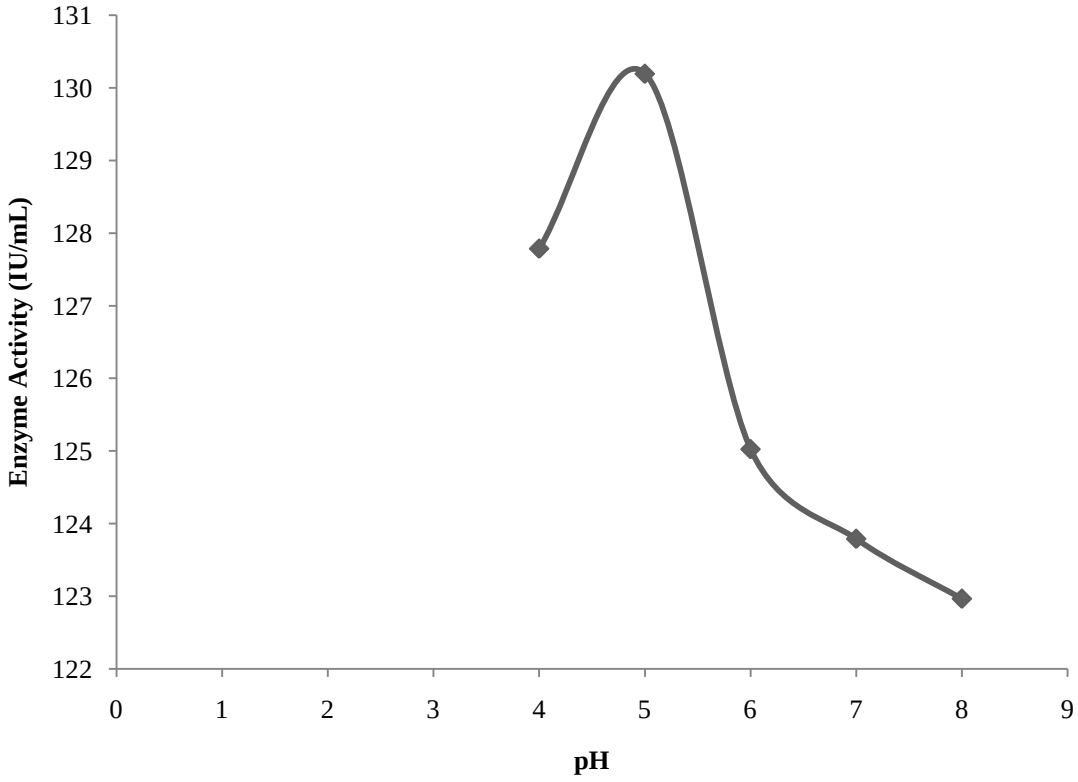

Figure 4.1: Effect of pH on Invertase Activity

Enzymes are affected by changes in pH (Campbell and Reece, 2002). Changes in pH can change the shape of the active site in an enzyme. Extremely high or low pH concentrations usually result in complete loss of enzyme activity due to denaturation (Helms, Kosinski, and Cummings, 1998). The effect of pH on invertase activity was determined at various pH from 4 to 8, using Acetate buffer (pH 4, pH 5), Phosphate buffer (pH 6, pH 7 and pH 8). The enzyme activity was measured at constant 55^{o}C for 1 hour and terminated it by heating in 100^{o}C water bath for 15 minutes.

As shown in Figure 4.1, invertase shows maximal activity at pH 5.0, which was in general range with many microbial sources invertase reported so far. The enzyme shows much more stability in acidic conditions than in basic. At pH 5.0, the enzyme shows high enzyme activity at 130 IU/mL. However, at basic conditions the invertase shows an abrupt decline in its activity. This result was supported by many author such as pH 4.6 to 6.5 by Boddy *et al*., 1992, pH 4.0 to 9.0 reported by Rubio *et al*., 1997, pH 3.5 to 5.5 by Nam, 2009, and pH 3.0 to 8.0 by Uma, 2010. The activity of invertase decrease drastically for pH more than 5.0.

The tendency for an acid or a base to be bound to an H^+ or to release an H^+ is influenced largely by [H^+] in the surrounding environment. If the [H^+] is high (low pH), then the side chain will tend to be bound to H+ so acidic side chains would be neutrally charged and basic side chains would be positively charged. This would dramatically alter the ionic interactions that could otherwise occur among acidic bonds. Furthermore, acids are normally weakly bound to one or more hydrogen ions, so that if dissolved in releasing one or more H^+ ions into solution. This could radically alter the structure of the protein. Since the catalytic ability of an enzyme is

so tightly linked to the specific shape and chemical properties of its active site, alteration tends to reduce catalytic function (retrieved from http://www.indiana.edu).

Extremely high pH do affect enzyme, as invertase could not carry out its physiological function such as hydrolysis of sucrose. This is due to the fact that invertase was denatured. The ionisation of side chains of the amino acids were affected by extreme pH values and the enzyme's active site also was disrupted thus the 3-dimensional structures was forced to change and not biologically active anymore (Campbell *et al.*, 1996).

4.2 Effect of Temperature on Invertase Stability

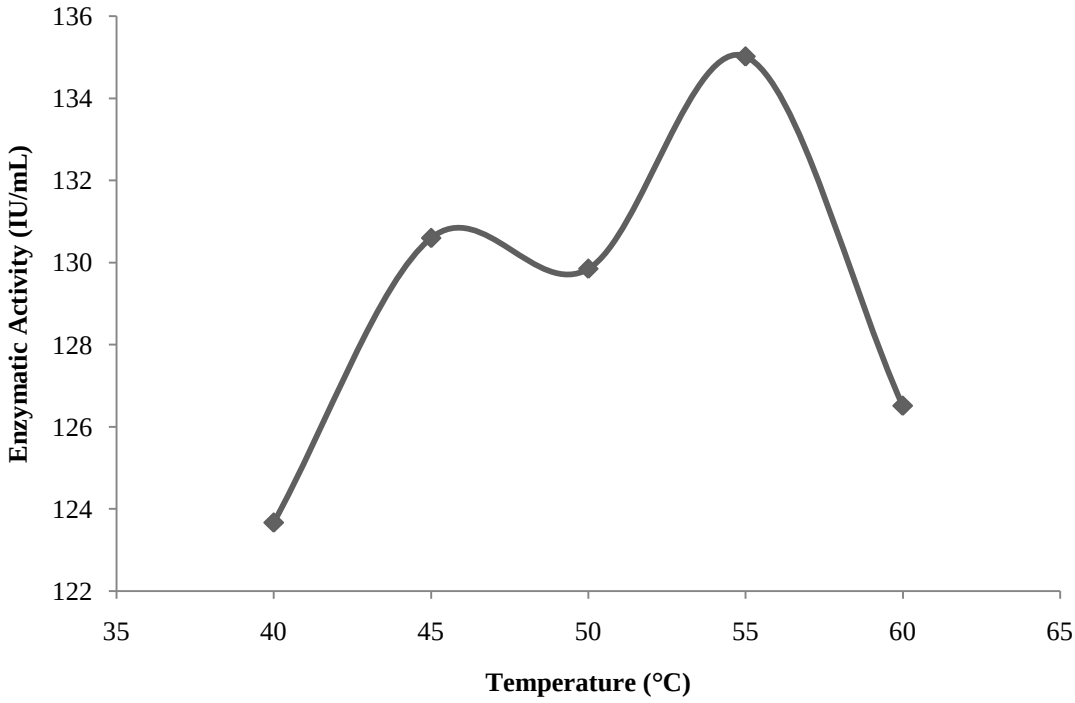

Figure 4.2: Effect of Temperature on Invertase Activity

The behaviour of invertase from fungal strain using sucrose as substrate was examined at constant pH 5.0 at different temperature from 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, and 60°C. From Figure 4.2, the graphs indicate a gradual increase from 40^{o}C to 45^{o}C, followed by slightly declined at 50^{o}C before slowly increased until 55^{o}C. At high temperature (60^{o}C) the invertase activity drastically decreased from 55^{o}C. From that, invertase was found to be active at 135 IU/mL when reaction mixture was kept at 55°C. This result was similar to that reported by L' Hocine *et al*. (2000) who found that the optimum temperature of *A. niger* invertase was 55°C. Compared to other authors, this result will be in range between 50^{o}C (Uma *et al*., 2010 and Boddy *et al*., 1993), 60^{o}C by María and María, 1995 and lowest values 30^{o}C reported by Rajoka and Yasmeen, 2005.

This result reflects the increase in invertase activity by temperature increment. As the temperature of the system is increased, internal energy of the molecules in a system will increase. Thus, the internal energy of the molecules including translational energy, vibration energy and rotational energy of the molecules involved in chemical bonding of the molecules as well as involved in nonbonding interactions. Some of this heat may be converted into chemical potential energy. If this chemical potential energy increases rapidly and great enough, then some of the weak bond that determines the dimensional shape of active site enzyme be broken. This could lead to a thermal denaturation of the enzyme and thus inactive it. However, decrease in amount of invertase activity present by denaturation that caused by too high temperature. So too much heat can cause the rate of an enzyme catalyzed reaction decrease because of the enzyme or substrate becomes denatured and inactive (retrieved from http://academic.brooklyn.cuny.edu/biology/bio4fv/page /enz_act.htm).

In general, high temperature is particularly interesting in industrial process; it would be preferable to use enzymes at high temperatures in order to make use of this increased rate of reaction plus the protection against microbial contamination and allows for greater substrate concentration. But, enzymes however are proteins and undergo essentially denaturation if it at temperatures above those they are ordinarily exposed in their natural environment. This decrease in activity was due to reduction in the enzyme stability (Russo *et. al*, 1996).

4.3 Determination of Kinetic Parameters

The Michaelis-Menten plot can shows the relation between the initial reaction rate (V) and the initial concentration of substrate (S). As in Figure 4.3.1, let V_{max} represent the maximum rate of reaction for total enzyme concentration, Michaelis-Menten equation become

$$v = \frac{V\max[S]}{Km + [S]} \tag{1}$$

At low substrate, $[S] \langle\langle Km$ equation 4.3 become

$$v = \frac{V\max[S]}{Km} \tag{2}$$

and the reaction is apparent as first order in substrate concentration. This means that the rate and substrate concentration are directly proportional to each other. Then, when high substrate concentrations, $[S] \rangle\rangle Km$ so the reaction turn to zero order with the equation which means the rate is equal to maximum velocity and independent to substrate concentration.

$$v = V\max \tag{3}$$

Consider when substrate concentration is such that the reaction rate is equal to one-half the maximum rate,

$$v = \frac{V\,\max}{2} \tag{4}$$

Therefore, Michaelis constant K_m value of the invertase was found to be 0.0333 g/L or 0.185 mM.

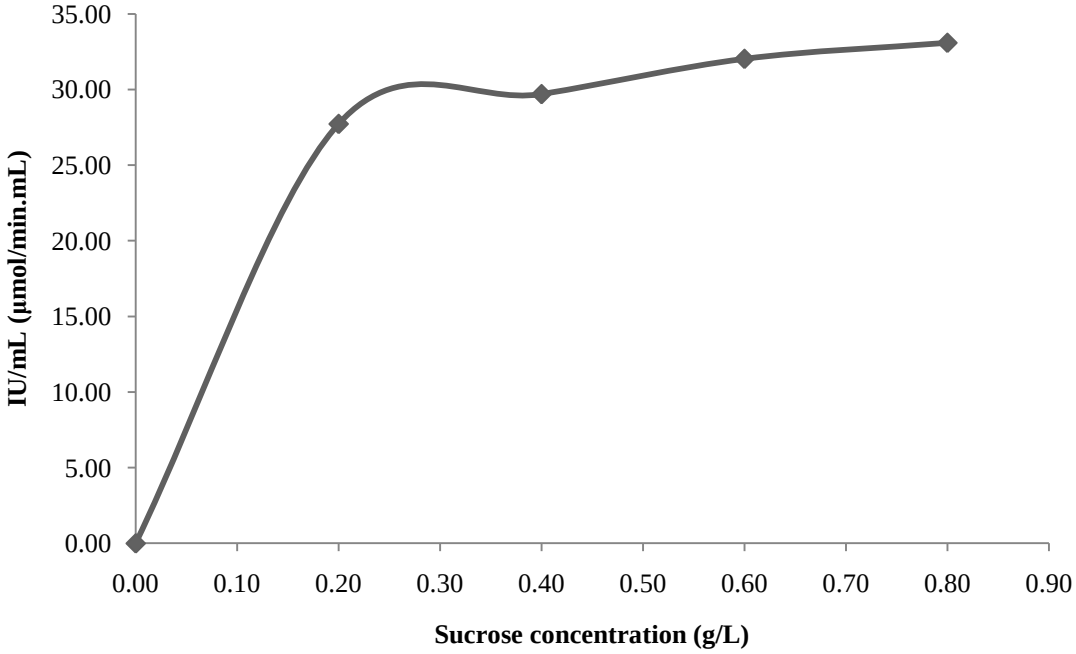

Figure 4.3.1: Michaelis-Menten Plot

By taking reciprocal of the Michaelis-Menten equation, we can obtain Lineweaver-Burk plot:

$$\frac{1}{v} = \left(\frac{Km}{V\,\max}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V\,\max} \tag{5}$$

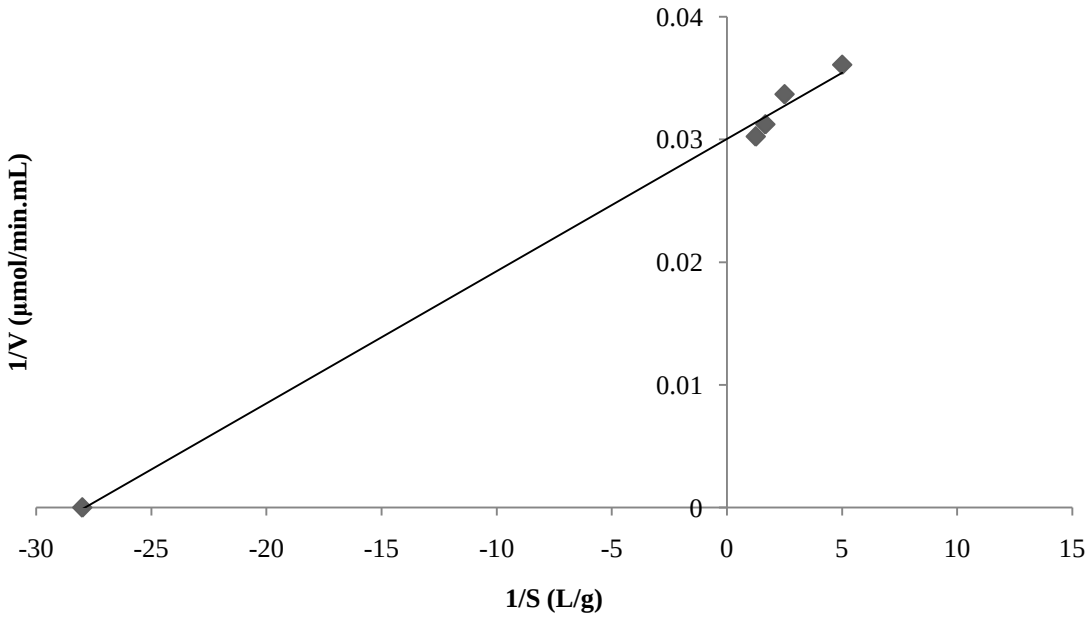

Figure 4.3.2: Lineweaver-Burk Plot

From Figure 4.3.2, V_{max} was 33.3333 µmol/min mL. This result is quite low compared to previous study which are 0.0625 mM and 1300 µmol/min (María and María, 1995), 35.67 mM and 398 µmol/min mL (L'Hocine *et al*., 2000) and 1.2 mM and 15800 IU/mL (Uma et al., 2010). This because maybe the concentration of sucrose used was not similar and their range is large. The V_{max} and K_m values increased significantly with the temperature for all enzymes (Stone *et. al.*, 2011). Particularly, when substrate concentrations are low, reducing K_m may be an effective strategy for microbes to increase enzymatic efficiency. The finding that K_m responds positively to temperature for hydrolytic enzymes is important because higher K_m values could offset increase in V_{max} with increasing temperature at low substrate concentrations (Davidson *et. al*., 2006).

The value of enzyme activity is generally highest when substrate concentration is unlimited. It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then increased, the

reaction velocity will increase too until it reaches its maximum. After this point, increases in substrate concentration will not increase the velocity (V_{max}). It is theorized that when this maximum velocity had been reached in a system, the entire available enzyme has been converted to enzyme substrate (ES) complex. This point on the graph is designated V_{max}.

4.4 Sucrose Utilization

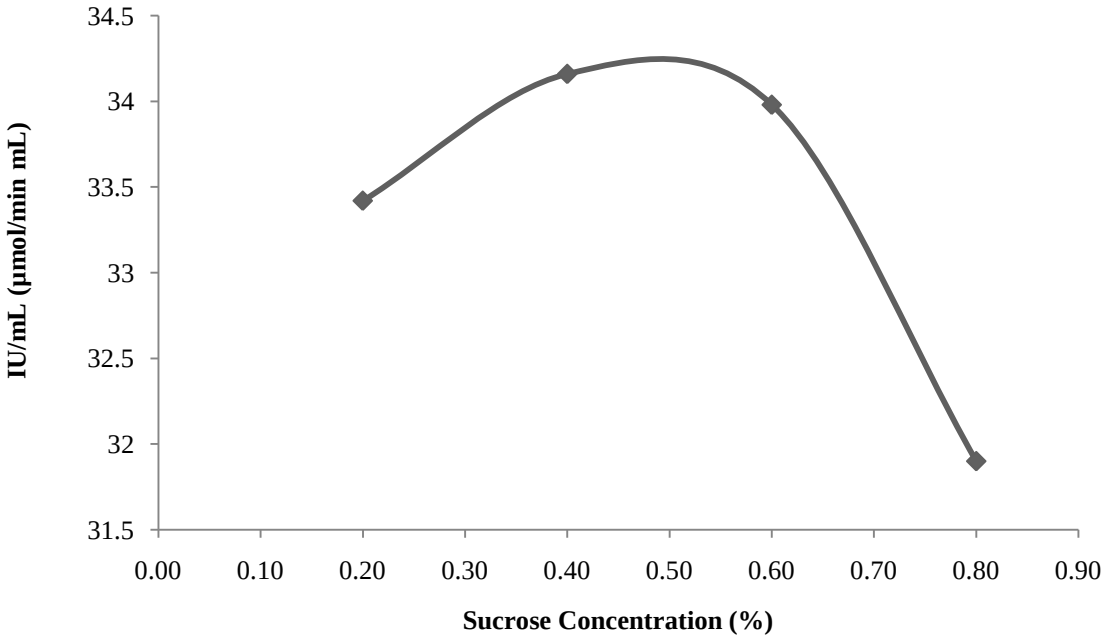

Figure 4.4: Effect of Sucrose Concentration on Invertase Activity

During microbial fermentation, carbon source is very important to provide energy source to cell. According to Cairns *et al*., 1995, invertase production by some other fungi was induced by sucrose. In order to study the possibility of sucrose as a carbon source for invertase production by *A. niger*, different concentration of sucrose

were used. As shown in Figure 4.4, the highest enzyme activity at 34.25 IU/mL was observed at 50% of substrate concentration. This result is higher than result from L'Hocine *et al*., 2000 which is 40% of sucrose concentration.

As sucrose concentration increased, the amount collisions between the substrate and the enzyme will increased too by assuming that all of controlling and environmental factors are kept constant, such as pH at 4.5 and temperature 55^{O}C. The number of collisions between the substrate and enzyme will increase, because of the collision theory, which states that some collisions indirectly cause the rate of reaction to increase. And thus increase the changes of an enzyme an enzyme-substrate complex formed. Increasing in substrate concentration may contribute to substrate saturation for enzyme invertase but until its reach maximum activity. L' Hocine *et al*., 2000 stated that at higher concentrations, the rate of reaction will decrease slowly probably due to decreasing in water activity.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The main objectives of this study which are to study the pH, thermostability and enzyme kinetics of invertase from *Aspergillus niger* were achieved. The pH and temperature optimum are 5.0 and 55^{o}C respectively. It shows the invertase is acid enzyme and need to be reacted in neutral condition which is 4.0 to 6.0. The enzyme kinetics also studied and based on the result, K_m is 0.0333 g/L and V_{max} 33.3333 µmol/min mL. Highest production on invertase was when substrate concentration at 50 g/L.

5.2 RECOMMENDATIONS

Some of recommendations are being proposed in order to produce high yield of invertase 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 enzyme concentration and incubation time in order to understand the effect of these parameters towards invertase stability.

2) Using other microbial organism

   Aspergillus niger has been used in this study to produce invertase. There are many more microbial organisms such as *Saccharomyces cerevisiae*, *Candida utilis*, *Azotobacter chroococcum* and *Lactobacillus reuteri* can produce invertase. Different microbial organism produce different yield of invertase.

3) Purification of enzyme.

   Invertase widely used in food industrial, so increase purification step in order to be more commercial purified enzyme. For suggestion on future work, addition of electrophoresis and determination of molecular weight by SDS-PAGE.

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APPENDIX A

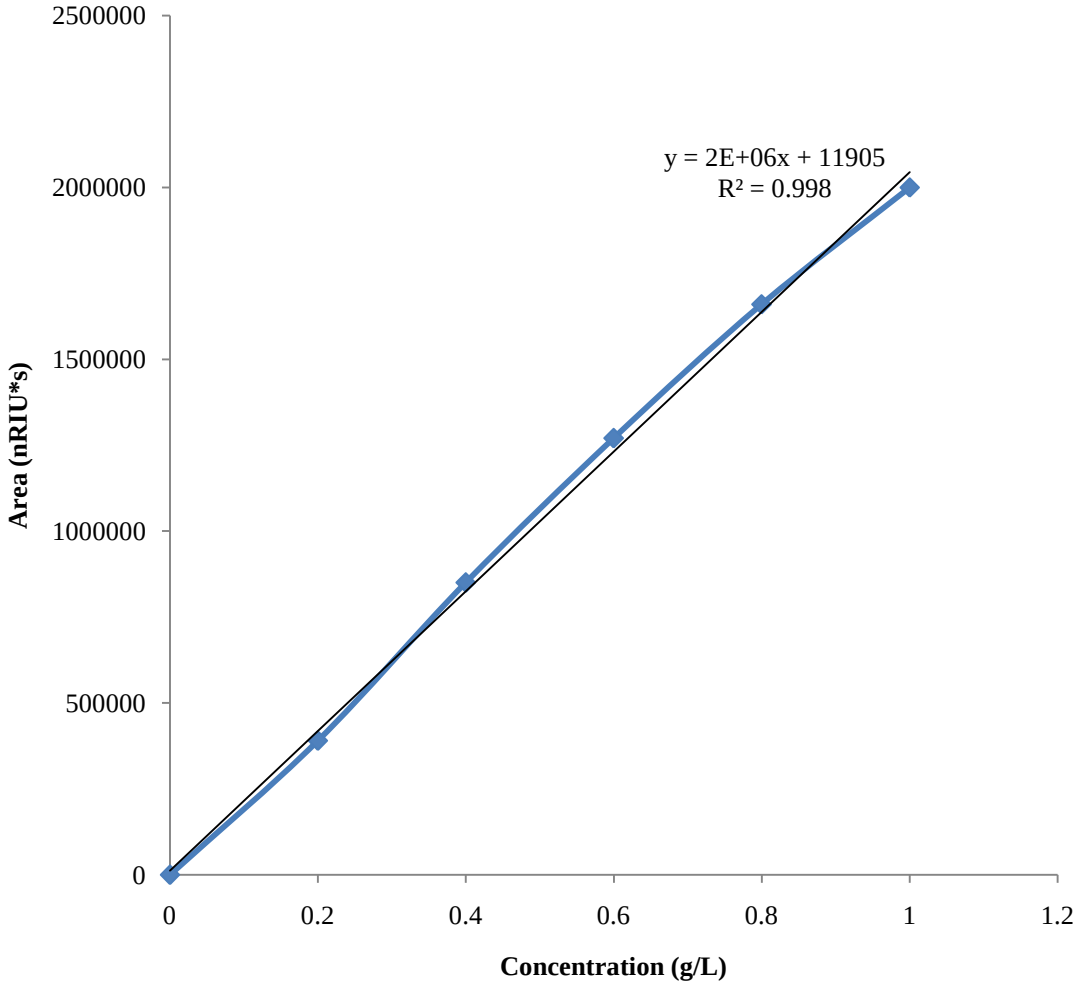

Figure A.1: Standard Calibration Curve of Glucose

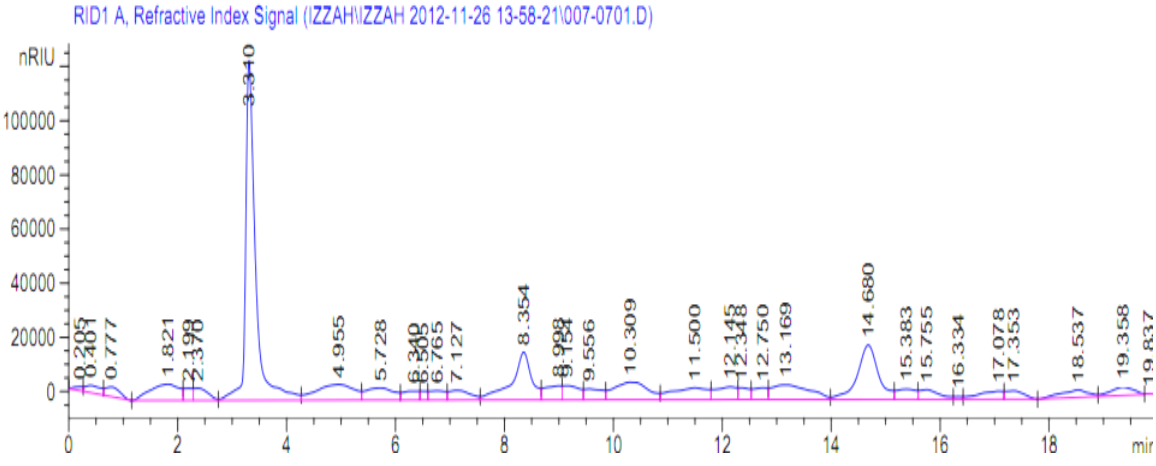

Figure A.2: HPLC Graph

Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	0.205	BV	0.1851	1.58341e4	1457.36230	0.2822
2	0.401	VV	0.2860	5.39870e4	2665.43701	0.9620
3	0.777	VV	0.3018	6.59939e4	3539.46704	1.1760
4	1.821	VV	0.5465	2.23427e5	6003.47754	3.9814
5	2.199	VV	0.1588	4.92894e4	4474.37646	0.8783
6	2.370	VV	0.2957	8.07762e4	4502.43945	1.4394
7	3.310	VV	0.1832	1.57577e6	1.25489e5	28.0796
8	4.955	VV	0.7286	2.80101e5	5789.78955	4.9913
9	5.728	VV	0.5386	1.60770e5	4444.60107	2.8649

Figure A.3: HPLC Result

Table A.1: Effect of pH towards Invertase Activity

Temperature (^{o}C)	pH	Peak Area (NRIU*s)	Enzyme (IU/mL)	Activity
55	4.0	1.39e^{6}	128	
55	5.0	1.42e^{6}	130	
55	6.0	1.33e^{6}	125	
55	7.0	1.35e^{6}	124	
55	8.0	1.34e^{6}	123	

Table A.2: Effect of Temperature towards Invertase Activity

pH	Temperature (^{o}C)	Peak Area (NRIU*s)	Enzyme (IU/mL)	Activity
4.5	40	1.35e^{6}	124	
4.5	45	1.42e^{6}	131	
4.5	50	1.41e^{6}	130	
4.5	55	1.47e^{6}	135	
4.5	60	1.38e^{6}	127	

Table A.3: Effect of Substrate Concentration on Incubation Time

Hour	0.20 g/L	IU/mL	0.40 g/L	IU/mL	0.60 g/L	IU/mL	0.80 g/L	IU/mL
0	$1.41e^6$	-	$1.35\ e^6$	-	$1.33\ e^6$	-	$1.31\ e^6$	-
4	$1.24\ e^6$	28	$1.33\ e^6$	31	$1.25\ e^6$	29	$1.33\ e^6$	31
8	$1.37\ e^6$	31	$1.22\ e^6$	28	$1.33\ e^6$	31	$1.32\ e^6$	30
12	$1.46\ e^6$	33	$1.49\ e^6$	34	$1.48\ e^6$	34	$1.39\ e^6$	32
16	$1.46\ e^6$	34	$1.29\ e^6$	30	$1.52\ e^6$	35	$1.27\ e^6$	29
20	$1.41\ e^6$	32	$1.26\ e^6$	29	$1.29\ e^6$	30	$1.24\ e^6$	28
24	$1.21\ e^6$	28	$1.29\ e^6$	30	$1.40\ e^6$	32	$1.44\ e^6$	33