CULTIVATING CONDITIONS INFLUENCE INVERTASE PRODUCTION BY *ASPERGILLUS NIGER* IN SUBMERGED CULTURE.

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

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ABSTRACT

Recently, there was increased in the production of microbial invertase due to its function which was important in production of invert sugar and high fructose syrup as compared to production of high fructose syrup and invert sugar for formulation of pharmaceutical product from sucrose by using acid hydrolysis. Therefore this study was conducted to examine the effect of substrate concentration by using sucrose from table sugar as cheaper carbon source of to provide an alternative production without implement other steps to secure the carbon source to be used in microbial invertase production by *Aspergillus niger*. This study was also conducted to study effect of pH and agitation speed on cultivating conditions by One Factor at a Time (OFAT) and eventually optimize the parameters for invertase production in submerged culture by using central composite design (CCD) in Design Expert for Response Surface Methodology (RSM) in submerged fermentation. The studied parameter were substrate concentration in selected range 10-50% (w/v), pH in range of pH 4.5-6.5, and agitation speed in range of 100-300 rpm. Studied conducted on one factor at a time yield maximum amount of invertase activity at 30 g/L sucrose concentration, pH 5.5 and 250 rpm with amount of 8.9132 IU/mL, 8.6754 IU/mL and 8.3429 IU/mL respectively after 40 hours fermentation period. After optimization of these three parameters using Response Surface Methodology (RSM), the optimum cultivating condition was obtained at 26.9583 IU/mL by using 30 g/L of sucrose concentration, pH 5.5 and 200 rpm. The optimum cultivating condition effect on invertase production in submerged culture was concluded at optimum condition obtain at 30 g/L sucrose concentration, pH 5.5 and 200 rpm from the study conducted by using central composite design since it had indicated that these three factors were significant with R^2 value of 0.9950 ($P<0.001$). Based on the result obtained from this study, it was recommended that table sugar can be used as carbon source and the model obtained can be further reviewed to ensure effective invertase production in submerged culture by *Aspergillus niger*.

ABSTRAK

Kebelakangan ini penghasilan invertase enzim daripada fermentasi oleh mikroorganisma semakin meningkat disebabkan oleh fungsi enzim ini yang dapat menghasilkan gula ringkas dan fruktosa berbanding penghasilan gula ringkas dengan menggunakan mekanisma penghasilan glukosa dan fruktosa daripada hidrolisis oleh asid. Oleh itu kajian dilaksanakan dengan meggunakan gula pasir sebagai substatu penghasilan invertase oleh *Aspergillus niger* dalam kultur kelalang bergoncang. Kajian juga dilaksanakan untuk mengkaji kesan pH dan kelajuan pengadukan terhadap penghasilan invertase dan seterusnya mengoptimumkan produktiviti dengan menggunakan rekabentuk komposit berpusat dalam perisian *Design Expert* untuk Kaedah Sambutan Permukaan oleh ferrmentasi di dalam media. Pembolehubah yang digunakan dalam kajian ini adalah 10-50(g/L) bagi kepekatan substratu, pH 4.5-6.5, dan kelajuan pengadukan dalam julat 100-300 rpm untuk kaedah satu faktor pada satu masa dan rekabentuk komposit berpusat. Keputusan yang optimum yang diperolehi untuk aktiviti invertase bagi kaedah satu factor pada satu masa adalah pada 30 g/L kepekatan sukrosa yang digunakan dalam medie, pH 5.5 and 250 rpm sebanyak 8.9132 IU/mL, 8.6754 IU/mL dan 8.3429 IU/mL bagi setiap faktor. Kaedah rekabentuk permukaan berpusat pula menunjukkan keadaan optimum yang boleh menghasilkan inverase adalah dengan menggunakan kepekatan sukrosa sebanyak 30 g/L pada pH 5.5 dan kejaluan pengadukan sebanyak 200 rpm kerana aktiviti invertase paling maksimum diperolehi dalam keadaan ini iaitu 26.9583 IU/mL dengan nilai R^2 iaitu 0.9950 ($P<0.001$). Konklusinya, keadaan optimum untuk menghasilkan invertase menggunakan kaedah fermentasi di dalam media ialah pada pH 5.5, 200 rpm dan kepekatan sukrosa sebanyak 30 g/L. Oleh itu disarankan kaedah fermentasi seperti ini yang menggunakan gula pasir dapat diguna pakai untuk menghasilkan enzim ini dengan membuat kajian lebih mendalam untuk penghasilan yang lebih efektif.

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X_3	Agitation speed of samples
B	Beta
X	Cell dry weight
b*ij*	Cross product coefficient
^0C	Degree Celcius
$	Dollar United States money
E	Error
Y	Invertase activity
$+\infty$	High factorial point
$-\infty$	Low level of factorial point
£	Model constant
%	Percentage
X_2	pH of samples
X_1	Sucrose concentration of samples

LIST OF ABBREVIATIONS

Adj R^2	Adjusted R^2
US $	United States Dollar unit
U/L.h	Units of enzyme per liter per hour
U/L	Units of enzyme per liter
U/g	Units of enzyme per gram
U	Unit of enzyme
Sp	Species
SHARP	Anomalous Patterson maps using subroutine XREP of program package SHELX 5.0 to refine the structure of invertase.
RSM	Response Surface Methodology
rpm	Revolution per minute
OFAT	One factor at a Time
NaOH	Sodium Hydroxides
NaCl	Sodium Chloride
mm	Millimeter
mM	Mill molar concentration
mL/min	Militre per minute
$MgSO_4.7H_2O$	Magnesium Sulphate Anhydrose
M	Molar unit concentration
LUNA NH_2 (AMINO)	Brand name of High Performance Liquid Chromatography Column.
KH_2PO_4	Potassium Dihyrogen Phosphate
HPLC	High Performance Liquid Chromatography
HCL	Hydrochloric Acid
GH32	Glycosides Hydrolyses 32 Family
FPLC	Fast Protein Liquid Chromatography
FOS	Fructooligosaccharides
$FeSO_4$	Iron (II) Sulphate
DOE	Design of Experiment
DMMULTI	Symmetry average initial phase in detection of solvent flattening invertase structure.
cm	Centimetre
CCD	Central Composite Design
ANOVA	Analysis of variance
Aa20	Strain number of *Aspergillus niger*
$(NH_4)_2 SO_4$	Ammonium Sulphate
(NH_4) CL	Ammonium Chloride
% w/v	Percentage of weight over volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Nowadays, there are high demand enzymes of food, drink, and confectionary industry. According to a fact from Enzymes for Food Processing Industry Project by Mott MacDonald, the author has reported that in the world enzyme market during year 2005 it is estimated about US $ 1000 million by the year which indicate that enzyme market offer high profit outcome and the market is continued growing .This increasing demand makes the requirement of enzymes production higher in industry. This requirement has developed much opportunity in industries regarding to market the potential way of production of enzyme with high profit income.

Invertase is a one of the beneficial enzyme that provides many products for industrial purpose such as pharmaceutical, food and etc. It is due to its utilization, its function to hydrolyze the sucrose into two equimolar mixtures of glucose and fructose at a concentration lower than 10 % sucrose (Guimaraes *et al.*, 2009). Therefore, this enzyme has attract researcher attention to study on any potential method of production that serves high effectiveness production method of microbial invertase although one previous method has been reported to produce this potential invertase in which the acid hydrolysis process but with low conversions efficiency which is 65-70% (Kaur and Sharma, 2005).

1.2 Problem Statement

Since microbial invertase production process is selected for this study, microorganism to be used in this study has become a serious matter to be observed. After some reviews, it is decided the *Aspergillus niger* is used to produce the invertase in submerged culture. However, there are many type of this species are discovered as the potential agent to produce this enzyme and therefore it is difficult to choose the most suitable type of *Aspergillus sp* for production process such as *Aspergillus niger* (Augur *et al*., 2000), *Aspergillus niveus* (Somera *et al*., 2009) and *Aspergillus fumigatus* (Gill *et al*., 2006). Therefore, the chosen type of *Aspergillus sp* must offer optimum production of invertase based on the effect of substrate concentration as well as the pH and the agitation speed on cultivating in submerged culture.

Problems arise from the substrate used in invertase production is one of the reason for this study to be conducted. It is because the local sucrose is expensive as compare to table sugar use as the substrate in this study. Therefore, new source of sucrose from table sugar in the market will be used to overcome this matter. The new selected sugar source should be able to produce higher invertase activity and cell biomass. Furthermore, since time is limitation factor to get the raw material, then this solution is used due to time saving method offered.

In fact, since the Response Surface Methodology is used in this study then time saving factor is become an important factor to be decided. Response Surface Methodology is more effective method to investigate the particular parameter that influences the invertase production in submerged culture compared to previous method that has been done by researcher before which is one-factor-at-a-time methods. This is because of the one-factor-at-a-time method is more time consuming and does not bring out the effect of interaction of various parameters (Elibol, 1999).

1.2 Research Objectives

The purpose of this study is to addresses an alternative method for production of invertase by selected microorganism and the utilization of cultivating condition for the growth of the selected microorganism.

The research objectives are:

1.2.1 To investigate the effect of substrate concentration (sucrose concentration), pH and the agitation speed on cultivating conditions for invertase production by *Aspergillus niger* in submerged culture.

1.2.2 To optimize the parameters for invertase production in submerged culture by using Response Surface Methodology (RSM).

1.3 Scope of study

This study is focusing on the scope as follows:

i. For the study of the effect of sucrose concentration influence the cultivating condition of invertase production in submerged culture by *Aspergillus niger*, a selected range of sucrose concentration is decided to be use from 10 until 50 % g/L.

ii. For the study on the effect of pH on the cultivating condition influencing the invertase production in submerged culture by *Aspergillus niger*, the range of pH use is pH 4.5, 5.0, 5.5, 6.0 and 6.5.

iii. For the effect of agitation speed that influence the cultivating condition of invertase production in submerged culture by *Aspergillus niger*, the range of rotation is selected in range 100 until 300 rpm.

iv. An optimization process of the parameters (sucrose concentration, pH and agitation speed) that influence the invertase production is conducted based on Response Surface Methodology (RSM) to achieve the most desirable condition of parameters for optimum level of invertase production.

1.4 Significance of Study

This study is believed to provide a new optimization method of invertase production by using microbial production method using *Aspergillus niger* with Random Surface Methodology (RSM) and provides a new alternative for raw materials of invertase production.

By using the Response Surface Methodology (RSM), a central composite design of experiment is used to investigate the optimum parameters (sucrose concentration, pH and agitation rate). This is because this method is more efficient compare to the using one-factor-at-a-time method that requires more times in conducting the experiment by shorting the time consuming for investigation of all parameters in this study.

Furthermore, a new potential source of raw material is evaluated for the purpose of producing lower cost invertase enzyme. Moreover, this raw material is easier than obtaining other carbon sources sucrose such using red carrot residue as substrate (Mona and Nooman, 2009) which require an extra method implemented in their production process to process the residue first before it is used as carbon source for invertase production. This will require more time to conduct the production of invertase enzyme and therefore it is wise to use local sugar as carbon sources that offer time saving method. Furthermore, this type of carbon source could be easily got in the market and cheaper than using commercial sucrose source from chemical company.

Moreover, this enzyme is widely used in confectionery industries and food industries. It is due to the characteristic that produce the fructose compound that have higher sweetening capacity, thus making this enzyme suitable for biotechnological applications, such as the production non-crystallizable sugars and soft centered chocolates (Rubio *et al.*,2003). It is also use to produce Fructooligosaccharides (FOS)

at higher concentrations of sucrose (Rustiguel *et al*., 2010). Furthermore, it has been used in the production of non-crystalling creams, jams, artificial honey and in confectionery industry (Emregul *et al*., 2007). Hence; study that related to production of this enzyme is encouraged.

In addition, this enzyme also can be used in pharmaceutical industry. It is useful for diabetics and potentiates iron absorption in children (Gill *et al*., 2006). Therefore, it is concluded that this study will be contribute large potential in market

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A review is performed to identify studies that relevant to this study cultivating conditions influence invertase production by *Aspergillus niger*. The following keywords has been used to identify the relevant material for this topic; function of invertase, microorganism that use to produce invertase, production of invertase, previous parameter use to investigate invertase production and analysis of invertase. This research is basically about the findings of an effective way to increase the invertase production in submerged culture. Therefore this chapter provides five major topic reviews with its own subtopics on function of invertase, microorganism that use to produce invertase, production of invertase, previous parameter use to investigate invertase production and analysis of invertase.

2.2 The Invertase Enzyme

It is the one of the earliest enzyme discovered by researcher which is isolated in the second half of the 19^{th} century and it is become a valuable enzyme due to its own function which is to produced "invert sugar" in ratio of product mixture 1:1 of mixture of dextrorotatory D-glucose and levorotatory D-fructose (1) (Alberto *et. al*, 2004). Therefore, there is crucial to recognize the structure and function of invertase.

Alberto *et al.* have been proposed research paper that stated the structure of invertase are in folded structure as illustrated in the Figure 2.1 which was established in year 2004. Based on the Figure 2.1, it is in a, ribbon structure which represented of the

monomeric unit of *T. maritime* invertase with the highlighted N-terminal β-propeller module and the five blades (numbered *I–V*), and the C-terminal β-sandwich module (dark red) in section A and the *B*-section of the figure represent the experimental map after phasing with SHARP (25), solvent-flattening with DMMULTI (26), and non-crystallographic symmetry and averaging with RESOLVE (27) for the experimental electron density map, contoured at a 1σlevel, shows three antiparallel β--strands in the β-sandwich module at the C-terminal region of the protein. These two structures is the experimental data presented by Alberto *et al.* in his paper.

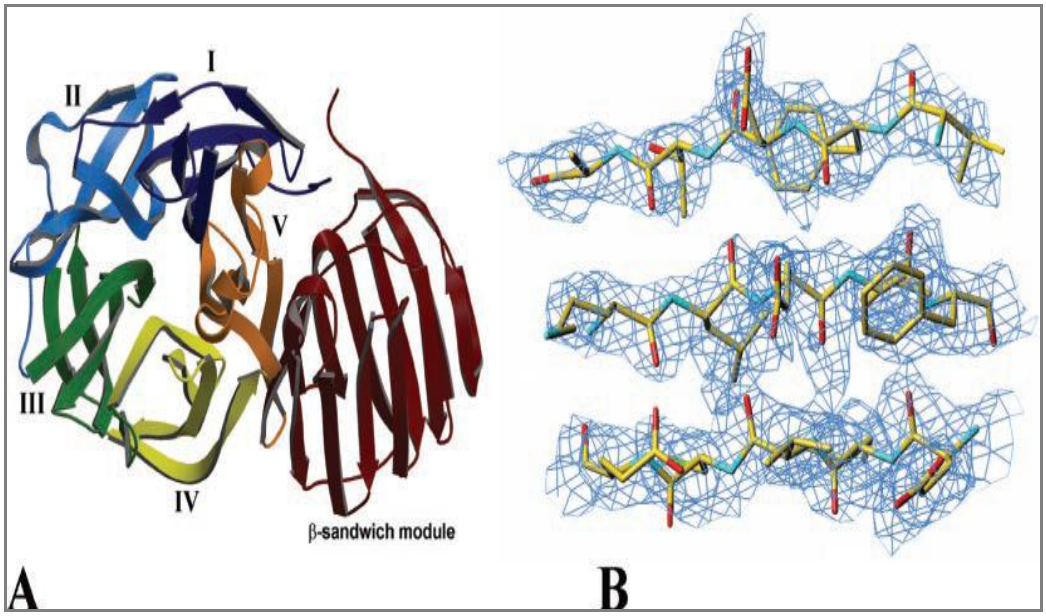

Figure. 2.1. The fold structure of invertase and experimental electron density map of invertase ribbon representation of the monomeric unit of *T. maritime.*

Source: Alberto *et al.*, (2004)

Since this type of enzyme is a high biotechnological potential β-D-fructofuranosidase, can be produced by many organisms, especially microorganisms like bacteria, yeast and filamentous fungi therefore it are produced in different formed by either intracellular or extracellular invertase (Rustiguel *et al.*, 2010).

This enzyme was not specifically defined in its structure until now. Researchers has proposed that a GH32 yeast invertase structure has not been reported until now, a

remarkable fact when taking into account that yeast invertase have been described as multimeric (Benito *et al.*,2010). Moreover, this research also proposed that the basic structural unit of intracellular and extracellular invertase is in dimer form as shown by electron microscopy but can be transformed into larger oligomers structure upon mannose binding (Benito *et al.,* 2007).

In fact, these enzymes have different structure or isoforms at different optimum pH but this different structure of enzyme were not reported to have specific function. It was only stated to have function as to control the entry of sucrose into different utilization (Alegre *et al*., 2009). According to Sturm in year 1999 as stated by Alegre *et al*. in year 2009, for acidic forms of invertase appear to have cell wall or vacuolar localization and structurally related to yeast and bacterial invertase. However, in neutral and alkaline forms were found in the cytosol (Vargas *et al*., 2003).

Further review lead to increase in understanding of invertase function. It is a member of GH32 family of glycoside hydrolases, which include more than 370 enzymes of vegetable and microbial origin. (Guimaraes *et al.*, 2007).It is function to hydrolyse the 1, 4-glycosidic bonds of sucrose and eventually formed equimolar mixtures of glucose and fructose that referred as invert sugar (Marquez *et al.*, 2008). It is a type of enzyme which is used for the inversions of sucrose in the preparation of invert sugar and high fructose syrup (Uma *et al.*, 2010).

Since it is naturally exist, therefore many researchers has been attracted to conduct a study on the contribution from this enzyme to the world nowadays. The Figure 2.2 represents the chemical structure of sucrose hydrolysis reaction catalyzed by invertase.

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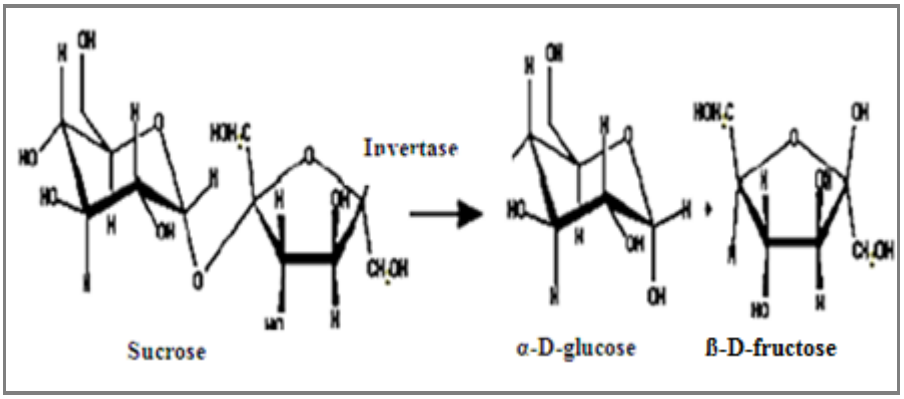

Figure 2.2: An illustration of chemical sucrose hydrolysis reaction catalysed by invertase

Source:**http://www.ensymm.com**. (Retrieved at 3 Nov., 2011)

Nowadays, this enzyme is widely used in confectionery industries and food industries. It is due to the characteristic that produce the fructose compound that have higher sweetening capacity, thus making this enzyme suitable for biotechnological applications, such as the production non-crystallizable sugars and soft centered chocolates (Rubio *et al.*,2003). Other than that, it has been used in the production of non-crystalling creams, jams, artificial honey and in confectionery industry (Emregul *et al.*, 2006).It is also used to produce Fructooligosaccharides (FOS) at higher concentrations of sucrose (Rustiguel *et al.*, 2010). Furthermore, it is also used for diebetics and potentiates iron absorption in children (Gill *et al.*, 2006). Therefore, it is concluded that this study will be contribute large potential in market.

2.3 The Microorganism use to Produce Invertase

This particular enzyme are produced from various type of microorganisms includes fungi, yeast and bacteria. The most type of microorganism that can produced this enzyme is fungi type such as *Aspergillus niger* (Ashokumar *et al.*, 2001), *Aspergillus niveus* (Guimaraes *et al*., 2009), *Aspergillus flavus* (Uma *et al.*, 2010), *Aspergillus phoenicis* (Rustiguel *et al.*, 2010), *Aspergillus fumigatus* (Uma *et al.*, 2010) and etc, and followed by yeasts such as *Saccharomyces cerevisae* (Mona, Nooman, 2009) and bacteria such as *Bacillus macerans* (Samia, 2006). Each type of microorganism requires a specific method to produce invertase enzyme and produced different level of enzyme production.

According to Ashokumar *et al.* in year 2001, by using submerged fermentation of *Aspergillus niger* strain the maximum result is obtained as 18.3 U/L.h for 120 hours fermentation time but the enzyme was optimized by undergo two step to yield 58.3 U/L.h . In addition, this paper also had conducted an investigation on optimization condition of invertase production by using solid state fermentation. As a result, there is existed of invertase productivity which was 81.8 U/L.h with less fermentation time that only required 72 hours yielding optimum productivity compares to submerged fermentation. However, this technique require an additional step compare to submerged fermentation because the sample need to subjected under mechanical squeeze to obtain the extract before the sample undergo the centrifugation step for extracellular enzyme purpose. Therefore, this method becomes undesirable for this research due to the limitation in time.

Other than the Ashokumar *et al.* research paper in year 2001 there are several research papers that used similar *Aspergillus niger.* They are Reddy *et al.* in his research paper in year 2010 and Rubio and Navarro 2006 based on their own method. According to Reddy *et al.* (2010), the investigation of parameters such as pH, temperature and different carbon and nitrogen sources obtain a maximum enzyme activity in 30.84 U/mL. The maximum enzyme activity is obtained at 96 hours fermentation time at pH 3.5 and 30^0C using basal medium that contains 2% molasses as carbon sources supplemented by 0.5 % soya bean meal.

However, it is differed according to Rubio and Navarro (2006) which is use to investigate the effect of raffinose, sucrose and turanose as carbon sources that obtain the maximum 4.0 U/mL enzyme productivity after 48 hours fermentation time. These indicate that the fermentation times are different under different cultivating conditions that influence the invertase production.

Besides the above strain, *Aspergillus niveus* is used to produce extracellular invertase by Guimaraes *et al.* in year 2009 under submerged fermentation. This research paper is conducted to investigate the effect of agroindustrial residues as carbon sources for invertase production. Based on this paper, it is discussed that the presence of sucrose in sugarcane bagasse contribute to the higher enzyme productivity compare to the presence of glucose which is stated that intracellular enzyme is the type of product that become greater compare to extracellular.

Aspergillus flavus is one of the *Aspergillus* strains which able to produce extracellular invertase. According to the Uma *et al.* in year 2010, this strain able to produce invertase and require four days to achieve optimum fermentation cultivating conditions at pH 5.0 and optimum temperature is 30^0C by using 3% inoculum size in Czapek Dox using fruit peel waste as fermentation substrate. This species is culture by using submerged fermentation method that enhance by the addition of sucrose and yeast extract for optimization purpose. Since this research use to purify the invertase therefore the optimum enzyme result is obtained in recovery process as 3.2 % and 5.8 fold.

Further review on the microorganism used to produce this enzyme introduces another *Aspergillus* species strain that able to produce this enzyme. It is *Aspergillus phoenicis* that use as a microorganism in Rustiguel *et al.* fermentation during year 2010. According to this research paper, this type of fungus was grown in Khanna medium that supplemented by wheat bran as carbon sources at temperature 40^0C for 72 hours to obtain the optimum fermentation result. This research paper is similar to the previous reviews which used to purify the extracellular enzyme product to yield 12.5 fold enzymes with 72% recovery. However, the optimum condition that reported is at temperature 60^0C and pH 4.5.

Aspergillus ochraceus is another strain that able to produce this enzyme, according to Guimaraes *et al.* in year 2007, this fungus is able to produce extracellular invertase at maximum yield for 2.68 fold after the purification process. This optimum result is obtained after 96 hours fermentation period at temperature 40^0C by using Khanna medium.

In contrast, the *Bacillus macerans* strain is also able to be used as a fermentation microorganism to produce this enzyme but it is undergo a more complicated fermentation process. It is used by Samia in year 2008 using repeated batch fermentation method. This research had used immobilized *Bacillus macerans* cells in calcium alginate and used for the production of invertase. The purpose of her research is to investigate the influence of alginate concentration, cation concentration, cell to alginate ratio, initial cell loading, curing time and bead diameter on conversion of sucrose to inverted syrup on fermentation product. She had used the immobilized cells in shake flasks study to consider the optimum parameter of her investigation. Finally, this researcher had proposed that the optimum parameter gained from the study to be 3% (w/v) sodium alginate, 3% (w/v) calcium chloride with 2 hours curing time, 200 alginate beads per flask with 2 mm bead diameter. This optimum parameter is based on fermentation of immobilized cells of *Bacillus macerans* in alginate beads that is suggested as more efficient for the production of invertase and can be reused for seven cycles (336 hours) without any loss in their activity and 12 cycles with 72% residual activity.

Other than that, *Saccharomyses Cerevisae NRRL Y-13632* is another microorganism that able to produce invertase. This is reported by Mona and Nooman in year 2009 in their research paper. Basically, this paper is discussed the cultivating condition under solid state fermentation for invertase production using food processing waste. The highest productivity of this research is 272.5 U/g for dry substrate used which is red carrot residues that is experimentally designed with seven nutrients component that contain (g %): $(NH_4)_2SO_4$, 4.5, KH_2PO_4,2.3, $FeSO_4$, 0.01, $MgSO_4.7H_2O$, 0.7, sucrose 5.0; urea,1.1; yeast extract 0.5 (pH 5.0) as described by Ashokumar *et al.* (2001). By undergo four days fermentation time the enzyme yields 29 fold as the optimum value. Besides, this research paper is introduced the pH range that

used to conduct invertase fermentation which is in range 5.0 until 7.0 with the optimum pH reach at pH 6.0 and temperature is 50^0C.

As conclusion, the *Aspergillus niger* strain is the type of species that is desirable for this submerged fermentation study due to the high productivity by using molasses that contains sucrose as the carbon sources which is similar to this research study. As compared to the other strain this species is normally produce higher amount of invertase value in the review of research paper and eventually consider as the most potential strain to be used as to investigate the optimum invertase production in submerged culture during this study.

All of those research papers are summarized in a Table 2.1 as follows for easier comparison purpose:

Table 2.1: Summarization of invertase production by using different microorganism

Species	References	Production
Aspergillus niger	Balasubramaniem *et al.* (2001)	The optimum result is 18.3 U/L.h for 120 h fermentation time but the enzyme was optimized by undergo two steps to yield 58.3 U/L.h.
	Reddy *et al.* (2010)	The maximum 4.0 U/mL of enzyme productivity yields after 48 h fermentation time.
	Rubio and Navarro (2006)	The maximum 4.0 U/mL enzyme productivity after 48 h fermentation time.
Aspergillus niveus	Guimaraes *et al.* (2009)	The optimum enzyme activity in amount of 30.84 ± 0.447 U/mL.
Aspergillus flavus	Uma *et al.* (2010)	The obtained result is 40.41 of total Unit of enzyme founded.

Table 2.1: Continued

Species	References	Production
Aspergillus phoenicis	Rustiguel *et al.* (2010)	Purified invertase at the optimum enzyme result is obtained in recovery process as 3.2% and 5.8 fold.
Aspergillus ochraceus	Guimaraes *et al.*(2007)	Produce extracellular invertase at maximum yield for 2.68 fold after the purification process.
Saccharomyces cerevisae	Samia (2008)	The highest productivity of this research is 272.5 U/g and enzyme yield is 29 fold after purification step.
Bacillus macerans	Mona and Nooman(2009)	Only indicated their result to be 72% residual activity.

2.4 The Process use to Produce Invertase

Reviews have been done to obtain information about the previous process that has been used to produce invertase enzyme. Based on several reports, there are some method has been reported on invertase production such as submerged fermentation, solid state fermentation, batch fermentation and etc.

Submerged fermentation is used by a group of researchers lead by Ashokumar *et al.*in year 2001 by investigating the optimum submerged media for invertase production. Although he has reported that the more efficient way to produce the higher invertase enzyme is by using solid state fermentation but he has suggested that the optimized media for submerged fermentation and solid state fermentation for the production of invertase have to be different (Ashokumar *et al.*, 2001). Based on this researcher report, it is stated that difference in optimum fermentation condition for each process. According to Ashokumar *et al.*, the maximum invertase productivity is 81.8 U/L per h is obtained in solid state fermentation for 72 h but 18.3 U/L per h in submerged fermentation for 120 hours. Each type of invertase production process chosen resulting in enzyme production eventually produce different of invertase activity yield. Therefore it is critical to use only specific composition of media for the fermentation of *Aspergillus niger* in order to produce desirable invertase enzyme

According to Aranda *et al.* report in 2006, during this researcher study there is 2.5 times larger invertase production in medium that contains glucose as repressor and sucrose as inducer compare to medium that without the present of sucrose. It is evaluated by using *Aspergillus niger* Aa-20 based on two type of substrate which was glucose and sucrose to investigate the induction-repression ratio. However, there is stated that until current research the difference of invertase production is unjustified. Therefore this study is alternative to be conducted in order to provide more information about the submerged fermentation of *Aspergillus niger* to produce invertase by using sucrose as substrate to investigate the function of sucrose in invertase production.

According to Neto *et al.* in year 1996, the invertase production is found to be at its specific activity at 2.70 U/g dry cell with the optimum condition for invertase

formation were: 2.55 L culture medium (yeast extract, 3.0 g/L; 5peptone, 5.0 g/L; glucose, 2.0 g/L; sucrose, 15.0 g/L; $Na_2HPO_4.12\ H_2O$, 2.4 g/L; $(NH_4)_2SO_4$, 5.1 g/L and $MgSO_4.\ 7H_2O$, 0.075 g/L); 0.45 L inoculum (0.70 g dry cell/L); pH = 4.5; T = 35 ^{0}C and DO = 4.0 mg/L. However, this method is undesirable since the aeration effect of invertase production is not a consideration to be accounted.

Based on those reviews therefore submerged fermentation is chosen as a desirable process to produce invertase for this study due to easier fermentation process condition.

2.5 The Investigation of Previous Parameter use to Produce Invertase

There are several parameters that have been investigated for invertase production which are effect of carbon source, effect of pH and effect of agitation speed.

2.5.1 The Effect of Carbon Source on Invertase Production

The use of sucrose to produce invertase also has become a matter to review since it is the choosen raw material for invertase production in submerged culture during this study. Uma *et al.* in year 2010 already investigate this type of carbon source as a factor that influence the invertase production by using *Aspergillus flavus* strain under optimized culture conditions on 4 days old of incubation at an optimum pH 5.0, 30°C and inoculums size 3% in Czapek Dox using fruit peel waste as a substrate (Uma *et al.*, 2001) and eventually enhance the production of this enzyme by adding sucrose and yeast extract as nutritional factor. Her finding is about the purified 5.8 fold with recovery of 3.2%. It is concluded that sucrose is one of the carbon source that can be used to produce invertase enzyme.

Furthermore there is research that proved on the effect of sucrose in production of invertase. According to Alegre *et al.*in year 2009, the extracellular invertase production is found to be decrease as addition of glucose but the intracellular production is enhanced to yield 3-5 folds. This result is used as the consideration for the reason of using sucrose in this research study.

Besides that the carbon source use to produce this enzyme is not limited. Some researcher has reported that the conversion on waste residue is useful for this enzyme production. In year 2009, further study on the method used to produce the invertase enzyme has become wide with a production process that involved intracellular and extracellular invertase reported by Guimaraes *et al*. in year 2007 .Through this paper, agro-industrial residues have now become the carbon source use under submerged fermentation producing different productivity between intracellular and extracellular enzyme glucose is reported to be desire carbon source to be used as the substrate that produce higher amount of intracellular invertase compare to extracellular invertase.

Moreover, red carrot residue is also contributed to the production of invertase enzyme. This is studied by using *Saccharomyces cerevisae* that has been selected as a microorganism use to study for invertase production. Researchers have selected this carbon source in his research and succeed to produce invertase with the highest level from the use of red carrot residue as substrate compared to using other food processing waste such as citrus waste, sugarcane bagasse, grape juice residue and apple pomace in which the productivity value is 272.5 U/g dry (Mona, Nooman, 2009). However this type of carbon source was not a comparable type of carbon source compare to the carbon source used in this study.

Guimaraes *et al.* in year 2007 has proposed that the highest invertase productivity is obtained by using sugarcane bagasse. It was extracellularly produced in Khanna medium at sucrose 83 totals U from 108 totals U under orbital agitation (100 rpm), at 40 ◦C, for 96 hours. The following Table 2.2 summarize the effect of carbon sources that influence invertase production which was grown in Khanna Medium.

Table 2.2: The Effect of Different Types of Carbon Sources Use For Extracellular Invertase Production

Carbon Source	ß-D-Fructofuranosidase activity (Total U)		Intracellular total protein (mg)
	Intracellular	Extracellular	
Deficient	1.8	4.2	0.86
Glucose	16.4	35.8	5.31
Sucrose	23.3	83.0	4.20
Cassava flour	1.9	22.8	1.17
Corncob	17.8	36.2	3.21
Crushed corncob	9.97	19.5	2.29
Oat meal	13.1	12.1	2.58
Rice Straw	2.1	19.1	2.05
Sugar cane bagasse	2.3	108.0	0.61
Wheat bran	16.2	20.3	1.44

Note: The fungus was grown in Khanna medium for 72 h, under agitation, at 40˚C, with the indicated carbon sources at concentration of 2% (saccharides) or 1% (agroindustrial residues). Total U: U/mL x volume of extract; total protein; mg/mL x volume of extract.

Source: Guimaraes *et al.* (2007)

An agroindustrial residue is one of the types of carbon sources that also used as carbon source to produce this enzyme. This type of agroindustrial residues is alternative carbon sources to produce invertase reported by Reddy in his paper in year 2010. This researcher has proposed that this alternative carbon sources also can be used to utilize this enzyme production by using *Aspergillus niger* PSSF21 and recommended as a promising strain able to produce this enzyme. This enzyme was obtain in 30.84 ± 0.447 U/mL at pH 3.5 and 30^0C using basal medium that contains molasses (20%) as carbon sources and use energy sources supplemented by soya bean meal.

2.5.2 The Effect of pH on Invertase Production

A review on the effect of pH as a factor influence the production of this enzyme is performed. In year 2005, researchers have reported the maximum production of invertase is at initial pH 6.0. This research has been done by Haq *et al*. which is use to investigated this factor as one of the factor that influence the effect of cultivation condition the affect the production of invertase by using hyperproducing *Saccharomyces cerevisae* isolates. However the highest growth is reported at pH 5.5.

Since the highest growth at pH 5.5 is decided to be a potential pH that can be used as the optimum pH for invertase production, now a literature survey on this pH level is reviewed. One paper has reported that at this pH 5.5, optimum invertase is produce by *Candida utilis* and stable at range between 3 until 6. In this study, this factor is investigated by using an appropiate dilution of the purified enzyme in a 0.1 M phosphate buffer (pH 5.1), and incubating for 40 min at different temperatures (40–80°C). Optimum pH for invertase activity was determined using the standard assay buffer (Montesino *et al*., 1992; pH 3–8). The effect of pH on enzyme stability was analyzed after incubation in buffered solution (pH 3–8) for 1 hour at 60°C. In all cases, the amount of glucose released was assayed as described before (Cha´vez *et al.*, 1997). Furthermore, this optimum pH is also reported by Nguyen in year 2005 which is stated that in the summary of the research paper that the enzyme is completely stable in the pH range from 4 up to 8 and has a pH optimum of 5.5 (Nguyen *et al.*, 2005).

2.5.3 The Effect of Agitation Speed on Invertase Production

The effect of agitation rate on invertase production is reviewed as a parameter for this study. Based on previous research paper, there are different values of agitation rate that has been used to produce invertase enzyme which are 100 rpm (Guimaraes *et al.*, 2009), 125 rpm (Uma *et al*., 2010), 190 rpm (Ashokumar *et al.*, 2001), 200 rpm (Romero *et al.*, 2000) and 200 rpm (Nguyen *et al.*, 2005). Each agitation speed above has been reported separately according to different research paper. Due to this, it is a wise initiative to evaluate a definite value of agitation rate for being use in the invertase production.

According to the first research paper wrote by Guimaraes *et al.* in year 2009 the invertase productivity is the 93.0 of total Unit of extracellular enzyme produced at 100 rpm, optimum temperature at 60^0C and pH 4.5. This enzyme is produced under submerged fermentation by using sugarcane bagasse as substrate. This result is the highest among the research paper that had been reviewed.

In addition the agitation speeds use to produce this enzyme at 190 rpm as used by Ashokumar *et al.* in year 2001 and 125 rpm for the agitation speed used by. Uma *et al.* in year 2010 suggested a different result in extracellular invertase product. According to Ashokumar *et al.* in year 2001, the optimum condition for extracellular invertase production is yield after 120 hours fermentation period which is in day five of fermentation with 58.3 U/L enzyme compare to the agitation speed used by Uma *et al.* in year 2010 which obtained the optimum results at day four in fermentation time with 284 total U of 3.2 % recovery.

However, these differences are obtained by using two differences *Aspergillus sp* strains which are *Aspergillus flavus* by Uma *et al.* in year 2010 but *Aspergillus niger* by Ashokumar *et al.* in year 2001 therefore there is needed for further verification of agitation speed effect on invertase production.

Other than that invertase was also produce in fermentation by using Basic Mineral Medium as stated by Romero *et al.* in year 2000 in his studies at 200 rpm

agitation speed in submerged fermentation of *Aspergillus niger*, the enzyme productivity obtained was 20 U/L. h. This enzyme was produced by using Basic Mineral Medium of fermentation at 30^0C under 200 rpm agitation speed for 72 hours. in contrast, according to Nguyen *et al.* in year 2005 which is also using the same agitation speed suggested a different enzyme production with shortens time that only 48 hours fermentation time to obtain maximum enzyme production with 50 % residual invertase activity by using same strain.

Based on these review therefore there is needed for further research to verified the optimum agitation speed that influence invertase production by *Aspergillus sp* in submerged culture.

2.6 The Previous Analysis of Invertase Production

Analysis of invertase activity is reviewed to obtain information that is related to this study. There many method that have been used before including Hi-load anion exchange chromatography (Hussain *et al.,* 2009 and High performance liquid chromatography (HPLC) (Guimaraes *et al.,* 2007) and etc.

Hi-load anion exchanged chromatography has been used by a researcher to conduct the analysis of invertase enzyme. At first, the step of analysis has begun with subjecting the partially purified invertase to FPLC Hiload anion exchange chromatography for further purification of this enzyme. Next, the invertase is has been eluted at about 640mM NaCl resulting 3.7 fold purified invertase and its recovery value about 62% recovery (Hussain *et al*, 2009). Therefore, this method of analysis is noted as a potential method to be selected as this study analysis method.

High Performance Liquid Chromatography (HPLC) has been used by another researchers to analysis its activity. The reviews are finally decided to use this model of analysis it is conducted by using basis of sucrose hydrolysis reaction. High performance liquid chromatography (HPLC) has been performed at 40^0C using a LUNA NH_2 (AMINO) with 5 µm particles and column dimension in 250 x 4.6mm and eluted by

80% acetonitrile as mobile phase and flow rate 1mL/min.This model of analysis is desirable analysis because of it is available for the purpose of this study.

Since this paper is discussed about analysis of the release of reduced sugar from sucrose hydrolysis by High Performance Liquid Chromatography (HPLC), therefore a type of column is review in purpose to detect the sugar composition release by invertase activity. According to a website that specifies an instrument to be used for sugar analysis recommended that a column named as LUNA NH_2 (AMINO) with 5 µm particles and column dimension in 250 x 4.6mm is examined as a potential column to be used in order to analyse sucrose hydrolysis product (www.instrument.com.cn ,retrieved at October, 25 2011). This column able to retain hydrogen-bonding compounds under three separation modes: Reversed Phased, Normal Phase, and Ion Exchange.

Furthermore, according to this website it is able to provide reproducible retention and selectivity with improved column lifetime. This type of column that use to operate based on Amino columns also can trigger a problem due to the bonded phase easily hydrolyses off the silica, shortening retention time over the life of the column. The bonded phase stability of Luna NH2 is illustrated by 1.5 to 11.0 pH stability and 100% aqueous mobile phase stability. Therefore, it is suitable to be used as sucrose hydrolysis product analysis in this study.

Moreover, this website also provides a sample of chromatogram for easier reference to be used. The chromatogram is in the Figure 2.3 as follows:

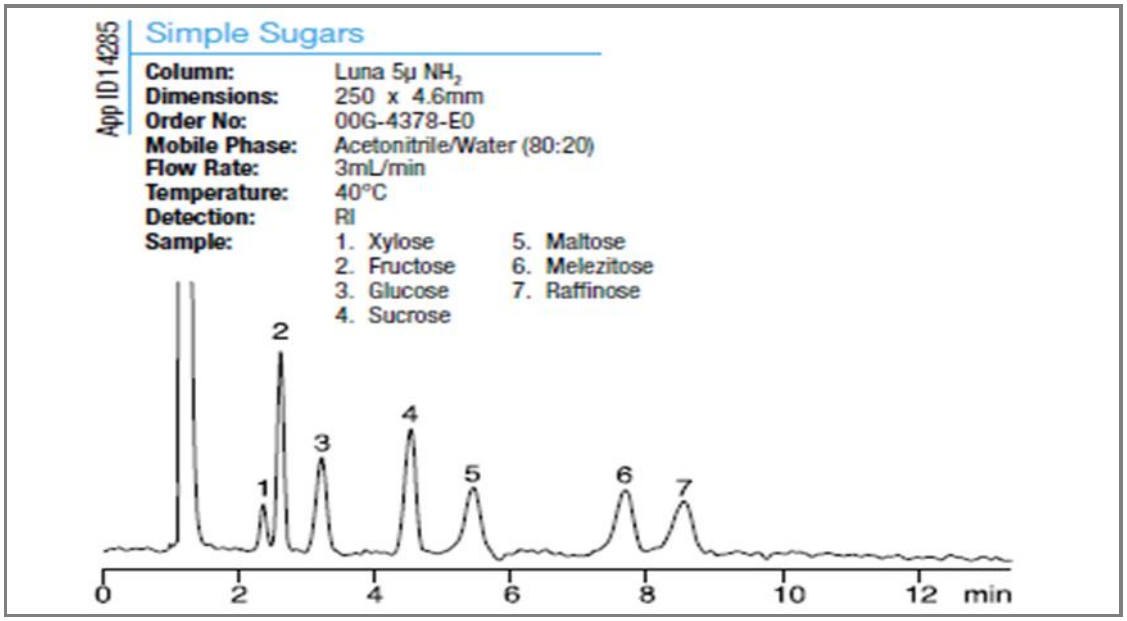

Figure 2.3: Chromatogram of simple sugar analysis using LUNA NH2 column.

Source:www.instrument.com.cn (Retrieved at October 25, 2011).

2.7 Optimization Method for Invertase Production

There was several design of experiment that was previously used to optimize the medium conditions for invertase production by using Design of Experiment software to copmpute the design. There method that was previously used was De Meo's Fractional Design, Box-Behnken, and One Factor at a Time (OFAT), Central Composite Design (CCD) and Taguchi Methodology as Response Surface Methodology.

Ashokumar *et al.* research paper in year 2001, the De Meo's Fractional Design has been used to obtain seven optimum component of medium that used to produce invertase under submerged fermentation and solid-state fermentation. Those components are Ammonium Sulfate, $(NH_2)_2SO_4$, Potassium dihydrogen Phosphate, KH_2PO_4, Iron II Sulfate, $FeSO_4$, Magnesium Sulfate anhydrous, $MgSO_4.7H_2O$, sucrose, urea and yeast extract. By applying this design of media the production of invertase in submerged fermentation can be increase to 58.U/L per h for optimum invertase activity compare to previous medium used before optimization process.

In addition, the design used by Ghasemi *et al.* in the research paper in year 2011 is different from previous design of optimization use by previous discussed paper. According to this paper, a Box-Behnken is used to identified and screened for optimum culture medium require for invertase production. Furthermore, this method is previously used by Plackett–Burman to production invertase in culture medium by using *Pichia sp.* This method is also represented the Response Surface Methodolgy. After using this design the invertase productivity has obtained an amount which is less than previous paper which is only 38.71 U/mL. Moreover, this model also proposed the optimal concentrations of sucrose, yeast extract and peptone in amount of 40, 5 and 4 g/mL, respectively that is 4 times higher than the original medium used.

Another optimization method used to maximize this enzyme production is Central Composite Design method use as Response Surface Methodology. This method has proposed by Songpim *et al.* in year 2011 by using *Candida guilliermondii* TISTR 5844 in submerged culture. The enzyme was studied by using four parameters which are pH, temperature, inoculum and substrate concentrations. Based on his study the maximum enzyme yields are 3197.34 U/L at pH 6.5, temperature 25^0C and 4.0 % inoculum in 200 mL fermentation working volume.

Taguchi Methodology is one of the Response Surface Methodolgy (RSM) used to study invertase production in submerged culture. This method is used to examine the relationship between the various components in the fermentation medium for optimization of component concentration in the medium by using the same strain of *Candida Guilliermondii* TISTR 5844 by Songpim *et al*. (2011). This method had considered three level factorials and represented by Songpim by the symbolic arrays *L9* in his paper which use to represent the primary information on the size of experiment. Each column in this compute design contains a number of conditions that depending upon level assigned by each factor. This study has used four variables and three concentrations levels. The variables used were inulin concentration, Ammonium Chloride (NH_4Cl), Magnesium Sulphate ($MgSO_4 \bullet 7H_2O$), and pH. This submerged fermentation has been conducted for 48 hours in 500 mL Erlenmeyer flasks with working volume 200 mL to yield maximum invertase at 838.20 U /L at pH 4,

concentration of inulin use is 50 %, concentration of Ammonium Chloride NH_4Cl is 4.8 %, and concentration of Magnesium Sulphate is $MgSO_4.7H_2O$ 0.6 %.

However, those productions were lesser if compared to a method used to produce invertase by using a bioprocess design of experiment using central composite design. Driouch *et al.* in year 2010 proposed that the achieved of optimized bioprocess for invertase production by using *Aspergillus niger*. It is because by using central composite design the extracellular invertase is able to achieve 400 U/mL in invertase productivity by using fed-batch bioreactor.

Therefore, an investigation of invertase production by using central composite design to investigate the effect of pH, substrate concentration and agitation speed by *Aspergillus niger* in submerged fermentation which used in this study is believed to provide a discovery of design of experiment method to optimize invertase production.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

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

3.2 Cultivation of Microorganism

Firstly, *Aspergillus niger* strain that was obtained from Malaysian Agricultural Research and Development Institute (MARDI) was cultured on potato dextrose agar medium at 30 °C and maintained at 4°C (Ashokumar *et al*., 2001). Then, *Aspergillus niger* will be used to prepared inoculum by transferring a loopful of spores from 3 days old slants *Aspergillus niger* into 250mL Erlenmeyer flaks containing sucrose 1% and yeast extract 1%(Nguyen *et al*., 2005). Next, it is incubated on a rotary shaker at 250 rpm, 30°C for 24 hours in 1g/L substrate (sucrose) in 250 mL Erlenmeyer flasks that was previously autoclaved. After that, the inoculum is subjected to production process which the fermentation of *Aspergillus niger* in 250 mL Erlenmeyer flasks.

3.3 Fermentation in Shake Flasks Culture

In this procedure, different variables were investigated with different manipulation within the range selected. Since there are three variables were used to investigate, therefore experiments will be conducted by manipulating one factor with the two variables constant. It will be conducted according to objectives this study. The effect of sucrose was investigated by using method as stated in the subtopics 3.3.1 which is the effect of sucrose concentration on invertase production, the effect of pH it was conducted by using method which was described in subtopics 3.3.2 which was the effect of pH, for the effect of agitation speed it was conducted by using method in in the subtopics 3.3.3 which was the effect of agitation speed and optimization of invertase production was conducted by using method described in the subtopics 3.4.

3.3.1 The Effect of Sucrose Concentration on Invertase Production

The fermentation medium for submerged culture consisted (g/L) of: $(NH_4)_2SO_4$-45; KH_2PO_4-23; $FeSO_4$-0.1; $MgSO_4 \cdot 7H_2O$-7; and yeast extract-1 % (Ashokumar *et al.*, 2001).Then, initial effect of sucrose concentration that influence the medium production of invertase is examined by using 10 g/L sucrose concentration in a flasks by constant pH at pH 5.0 in 1M sodium hydroxides (NaOH) at 30°C and 250 rpm.

24 hours old inoculums was transferred to 100 mL fermentation medium in flasks and subjected to incubation on rotary shaker at 30°C with agitation speed at 250 rpm (Sangeetha *et al.*, 2005). After that, the cultures were harvested at regular time intervals and assayed of the enzymatic activity, biomass and sucrose was analyzed.

In order to study of the effect of sucrose concentration on invertase activity the sucrose concentration was increase by 10%, 30% and 50% to investigate their effect on invertase production that is previously reported by Sanchez in year 2010 by modified range of sucrose concentration to this study range which is 10 g/L, 30 g/L and 50 g/L.

3.3.2 The Effect of pH on the Invertase Production

The effect of pH 4.5 was studied by adjusting pH of the medium with 1.0 M Hydrochloric Acid (HCl) and 1.0 M Sodium Hydroxide (NaOH) solutions. Invertase production was carried out in 250 mL flask with working volume 100 mL incubated at 33^0C for 48 hours. Sample were taken at different time interval and analyzed for invertase activity.

3.3.3 The Effect of Agitation Speed on the Invertase Production

The effect of initial agitation speed 100 rpm was studied by using constant the pH at 5.5 and sucrose concentration at 30 g/L of the 100 mL working volume fermentation in 250 mL Erlenmeyer flasks at temperature of 33^0C. Sample had been taken at different time interval and analyzed for invertase activity. This procedure was repeated for 200 rpm and 300 rpm to study the different used of agitation speed on invertase production in submerged culture.

3.4 Optimization of Medium for Invertase Production

Optimization of invertase production was done by using Response Surface Methodology method to find the optimum sucrose concentration, pH and agitation rate cultivating condition influencing invertase by *Aspergillus niger* in submerged culture. This study was done by using Central Composite Design (CCD) in Design Expert 6.0.8 to study the interaction of process variables for RSM.

The optimal invertase productivity was analyzed based on selected fermentation conditions such as pH (pH 4.5, 5.5 and 7.5); substrate concentrations (10 g/L, 30 g/L and 50 g/L) and agitation speed (100 rpm, 200 rpm and 300 rpm) were optimized. Each

of these variables is varied over five levels which were low axial point (-∞), low factorial point (-1), central point (0), high axial point (+∞) and high factorial point (+1). Table 3.1 shows the list of range and coded level of fermentation process variables. The total number of experiments was 20. Table 3.2 shows the design matrix of CCD for extracellular invertase production. Invertase activity (Y) and cell dry weight (Y_2) were taken as the response of the design experiment. The full quadratic equation of the response variables for invertase production was derived by using RSM as equation (3.1).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{21} + b_{22}X_{22} + b_{33}X_{23} + b_{12}X_1 X_2 + b_{13}X_1X_3 + b2_3X_2 X_3 + £ \quad (3.1)$$

Where Y = invertase production (U/L) or response, bi = the linear coefficients, bii = the quadratic coefficient, bij = the cross product coefficients and £ = the model constant. X_1, X_2, X_3 =Variables.

Table 3.1: Experimental range and level coded of process variables

Independent variables	**Units**	**Symbol**	**Ranges and levels**				
			-∞	-1	0	+1	+∞
Sucrose concentration	g/L	X_1	3.64	10.00	30.00	50.00	63.64
pH	-	X_2	3.82	4.50	5.50	6.50	7.18
Agitation speed	rpm	X_3	31.82	100.00	200.00	300.00	368.18

Table 3.2: Experimental design of CCD for extracellular invertase production

Run	X_2 Substrate Concentration (g/L)	X_1 pH	X_3 Agitation Speed (rpm)
1	50.00	4.50	100.00
2	30.00	5.50	200.00
3	30.00	5.50	200.00
4	10.00	6.50	100.00
5	30.00	5.50	200.00
6	50.00	4.50	300.00
7	10.00	4.50	100.00
8	30.00	7.18	200.00
9	30.00	5.50	31.82
10	30.00	5.50	368.18
11	30.00	5.50	200.00
12	30.00	5.50	200.00
13	10.00	6.50	300.00
14	63.64	5.50	200.00
15	10.00	5.50	300.00
16	30.00	5.50	200.00
17	50.00	6.50	300.00
18	50.00	6.50	300.00
19	3.64	5.50	200.00
20	30.00	3.82	200.00

3.5 Analytical Methods

Finally, data analysis was done by using two different methods which are cell dry weight and invertase activity by using High Performance Liquid Chromatography. Since the quantification of reduce sugar release by invertase activity was analyzed therefore a section was provided to be used to prepared a standard method for calibration of glucose concentration release by invertase activity.

3.5.1 Determination of Glucose Standard Curve

During this step, calibration curve of glucose concentration by using five different concentration glucose solutions which were 0.0 ppm, 0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm and 1.0 ppm. These standard solutions were then analyzed by using HPLC to obtain result for calibration that used as a reference for the release of glucose concentration by invertase activity.

3.5.2 Determination of Invertase Activity

The enzymes were centrifuged at (5000 x g) at 4^0C for 15 min (Rustiguel *et al.*, 2010) to obtain supernatant that consists of invertase enzyme. Next, the 0.5 mL of supernatant was mixed with 2.5 mL sucrose (60 g/L) and 0.5 mL of 0.1 M acetate buffer in a test tube. Then, test mix sample is subjected to incubation in water bath at 55^0C for one hour. After that, the reaction was stopped by subjected into boiling water for 10 minutes.

This enzyme activity was measured by estimation the releasing of reducing sugars from sucrose hydrolysis according to method used by Rubio and Navarro in 2006. Based on this article one enzyme unit was defined as the amount of enzyme need to produce one μmol of reducing sugar per minute and as described by Chen in year 1996 that was used as reference for Rubio and Navarro in year 2005 a specific enzyme production was defined as amount of enzyme units per milligram of dried biomass.

According to Sanchez *et al.* in 2010, one unit of invertase enzyme was defined as the amount of enzyme requires releasing 1μmol of glucose per minute. According to this paper the enzyme activity was calculated by using specific formula in which per reaction volume was the β-D-fructofuranosidases. The formula used was as follows:

$$\text{Units/mL} = \frac{\text{μmol glucose released}}{\text{Incubation time (min) x Volume assays (mL)}} \qquad (3.2)$$

3.5.3 Determination of Cell Concentration

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

$$\text{X (g/L)} = \frac{\text{[(weight dry filter paper + cell)-(weight of dry filter paper)] (g)}}{\text{Sample volume (L)}} \qquad (3.3)$$

3.5.2 Analysis of Invertase Enzyme by Using High Liquid Performance Chromatography (HPLC)

Sucrose hydrolysis of invertase is used as a basis of enzymatic assays by using HPLC. It was analyzed based on refractive index of product that will be formed after sucrose hydrolysis process. The Agilent series High Performance Liquid Chromatography (HPLC 1200) that equipped with LUNA 5 µ NH_2 25 cm x 4.6 mm column was operated by using acetonitrile as the mobile phase in ratio 20:70 water: acetonitrile as mobile phase, mixed with ultra - pure water and column temperature was maintained at 40^0C. The flow rate used was 1mL/min. (Rustiguel e*t al*., 2010).

3.6 Flowchart of Invertase Production by *Aspergillus niger* in Submerged Culture

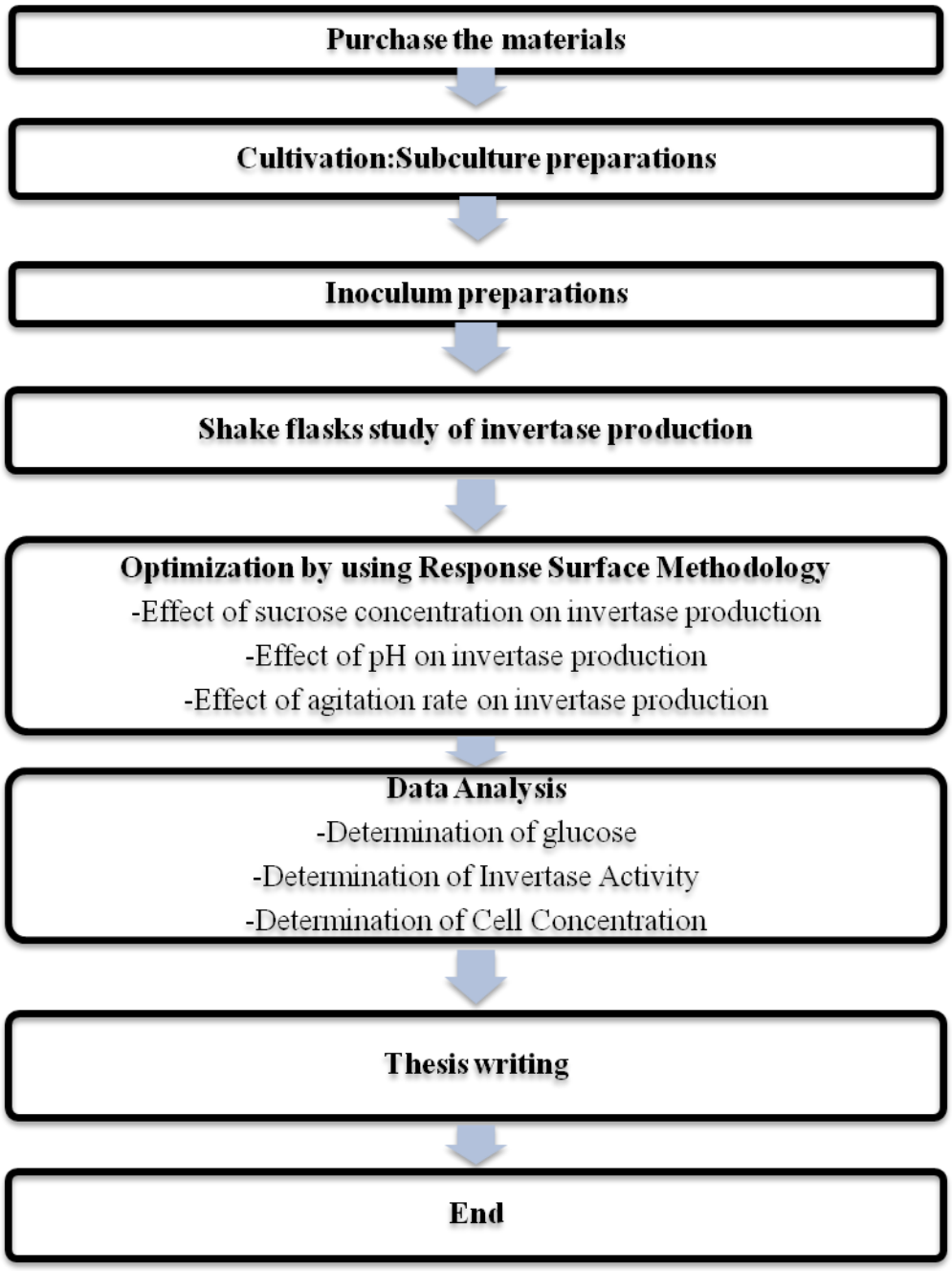

Figure 3.1: Flowchart of invertase production by *Aspergillus niger* in submerged culture.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

This chapter is discussing the result which was obtained during this study based on the effect of cultivating conditions for each parameters selected on invertase production in submerged species by *Aspergillus niger* in submerged culture which were sucrose concentration, pH and agitation speed. The data obtained was presented in subsection as cell dry weight of invertase enzyme, one factor at a time for effect of sucrose concentration, pH and agitation speed and optimization of the invertase enzyme production that measured based on invertase activity of the biomass.

4.2 Cell Dry Weight of Invertase Enzyme

This section present the data obtained from the two days of fermentation of *Aspergillus niger* in submerged culture, the result obtained was used to examined the growth profile of *Aspergillus niger* in the medium conditions by plotting the cell dry weight value against harvested time invertal. Relationship of this cell dry weight with the invertase production was examined by plotting invertase activity at a secondary axis of the same graph against the time of the fermentation. The data were presented in Appendix C and Figure 4.1 as followed.

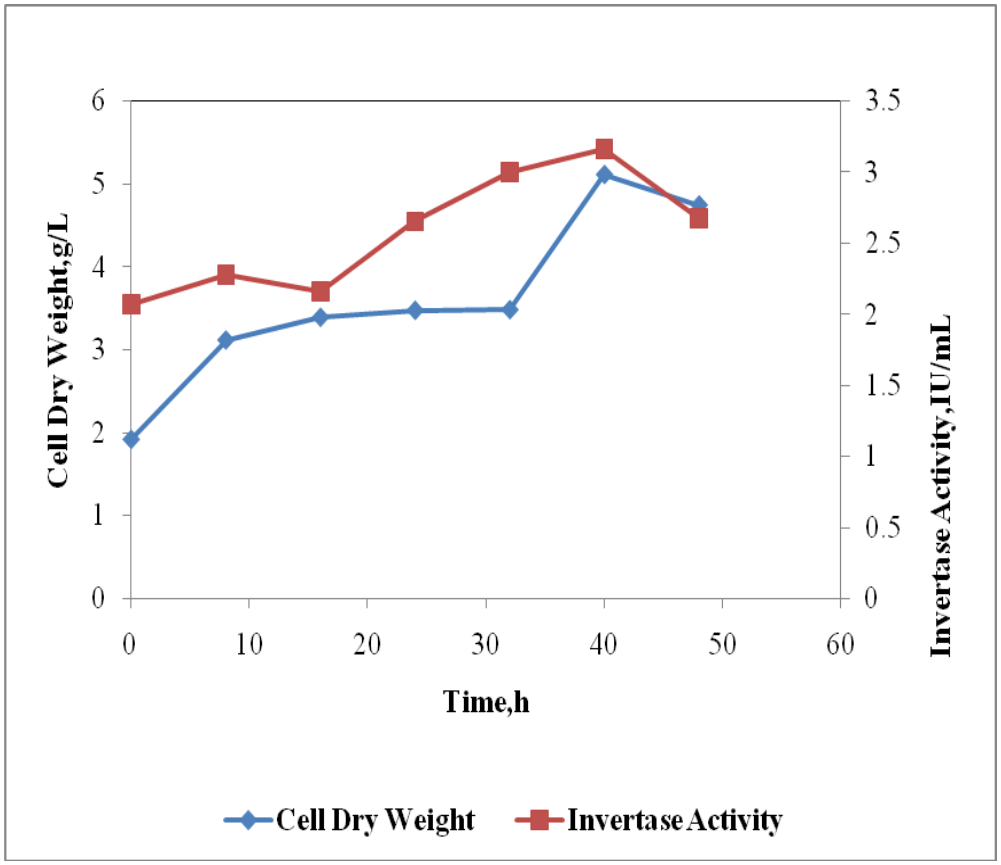

Figure 4.1: Growth profile of *Aspergillus niger* and invertase enzyme activity.

The cell dry weight data was analyzed at regular time interval at every eight hours starting from 0 to 48 hours. The sample then centrifuged to obtain the biomass by filtration before it was dried overnight at 80^0C in oven. After that, the weight of dry filter paper with dry cell was weighted to determine the cell dry mass. Final data was analyzed and presented as the Figure 4.1 above. Based on the Figure 4.1 above the biomass shows a growth profile curve within the fermentation medium.

Initially the *Aspergillus niger* exhibit a lag phase from 0 to 16 hours, it was because during this period it still started to increase in cell dry weight which shows that it was having an adaptation process with submerged medium. After 16 hours, it was clearly shows that significantly increase in dry cell mass. It is called as exponentially phase or log phase in which this microb started to grow until it reach maximum growth. The stationary phase is reach at 40 hours fermentation period and finally decreased at 48 hours fermentation time.

Meanwhile the supernatant obtained from the centrifugation step was analyzed for extracellular invertase enzyme production which measured in invertase activity was presented in the Figure 4.1 above to examine the relationship between the dry cell mass and invertase production. It was clearly shows that the growth of this fungi was a growth associated with the invertase production in which invertase enzyme was maximumly produced at highest cell concentration. Therefore, the further investigation was conducted for 40 hours to obtain optimum invertase enzyme production.

4.3. One Factor at a Time

This step was conducted in shake flasks study for 40 hours period of fermentation time at 30^0C by manipulating one independent variable while fixing other variables at certain level. The variable or process parameters involved were sucrose concentration from table sugar, pH and agitation speed .

4.3.1 Effect of Substrate Concentration on Invertase Production in Submerged Culture

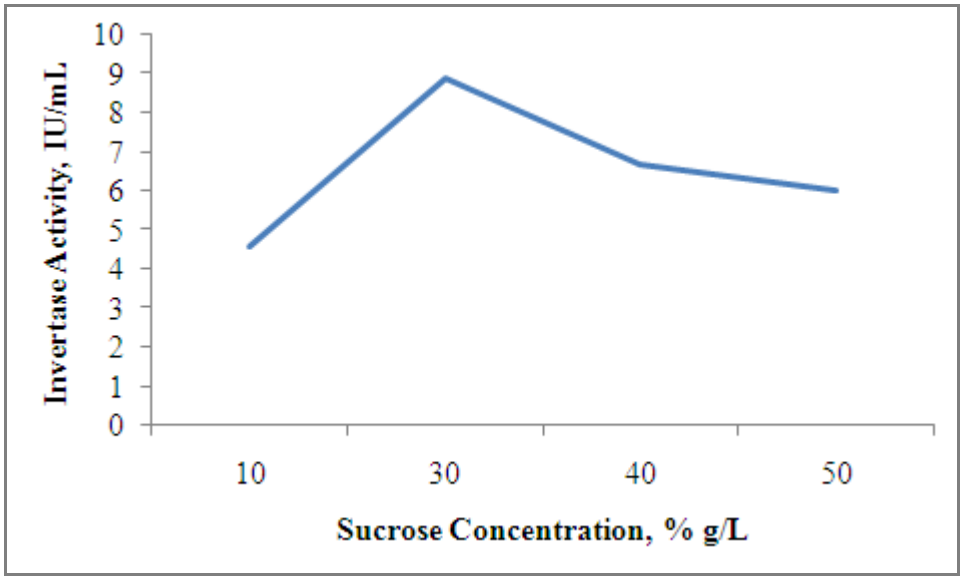

Figure 4.2: Graph of the effect of sucrose concentration on invertase activity.

The effect of sucrose concentration (10-50 g/L) on invertase production was determined. Based on the Figure 4.2,the maximum invertase activity is obtained by using 30 g/L of sucrose concentration at 30^0C, 250 rpm and pH 5.5.

Besides that, Goksungur *et al* in year 2005 reported that inhibition of invertase production might occur due to disaccharide characteristic of sucrose. Therefore, concentration of substrate in medium causing initially increase in range 10 g/L to 30 g/L and a reverse effect was observe after maximum level of concentration was achieved and the maximum invertase activity yield in this study was 8.9132 g/L at 30 g/L and started to declined in medium that contained 30 g/L sucrose concentration.

The similar trend was also stated by Siqueira *et al.*, in year 2008 which the reverse trend may be attribute to high sugar content . As a result, there was decreased in water activity as sucrose was a disaccharide that can contribute to increasing its osmotic pressure and causing inhibition to the cells cause eventually causing the decreased in invertase production (Siti Hatijah, 2010). Therefore, it shows that the preferable

conditions for this enzyme production was 30 g/L as the maximum invertase activity was detected by using High Performance Liquid Chromatography in medium that contain this amount of substrate.

4.3.2 Effect of pH on Invertase Production in Submerged Culture

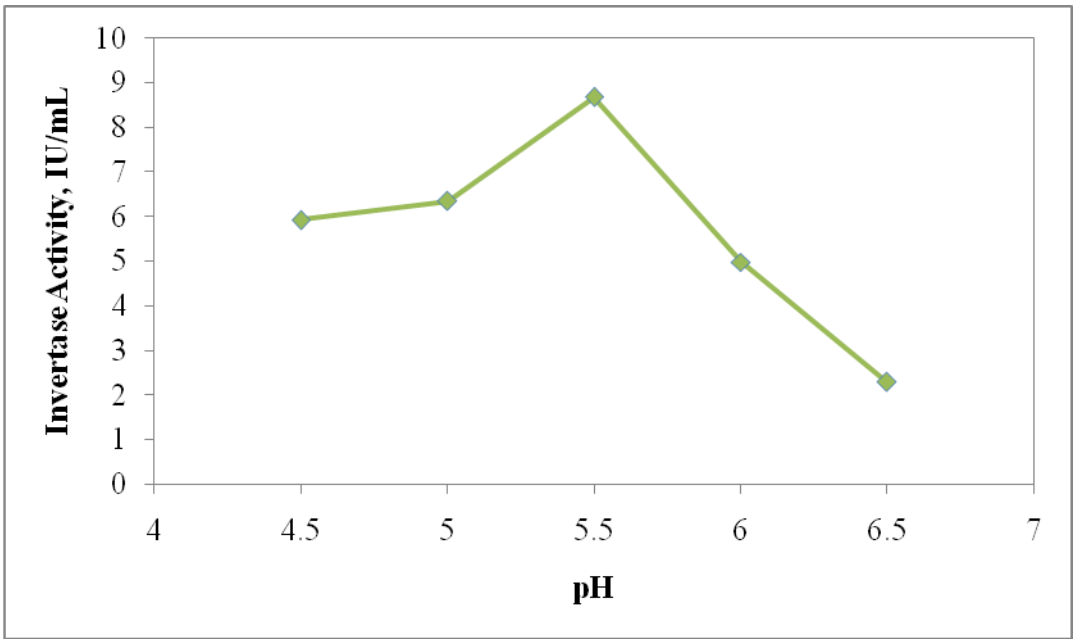

Figure 4.3:Invertase production influence by pH of growth medium.

Invertase production can be influenced by pH in the cultivating condition. To investigate the effect of pH in the fermentation medium to the invertase production the range of pH from 4.5 to 6.5 was used as the pH in shake flasks study. The data obtained were presented in Table 4.3 in Appendix E and Figure 4.3 to show the interaction of pH with invertase production.

Based on the Figure 4.3 ,there were significantly increased in invertase activity started at pH 4.5 to 5.5 and decreased after pH 5.5 which is to a lowest enzyme activity at pH 6.5. This trend indicate that the characteristic of this enzyme was stable at range of pH in the medium from pH 4.5 to 6.5 but the maximum invertase activity was obtained pH 5.5 which was similar to the optimum pH obtained by Nguyen *et al.* in year 2003 at 96.50 U *Aspergillus niger* IMI303386 but it was different to maximum invertase

activity obtained by production of invertase by *Saccharomyses cerevisiae* which is also a fungal type microorganism that exhibit maximum invertase activity at pH 6.0 at 272.5 U/g of dry substrate concentration with 72 % residual activity (Mona and Nooman,2009).

However, the effect of initial pH was similar to the effect of invertase production influence by pH in solid state fermentation medium obtained by Sangeetha *et al*. in year 2005.

4.2.3 Effect of Agitation Speed on Invertase Production in Submerged Culture

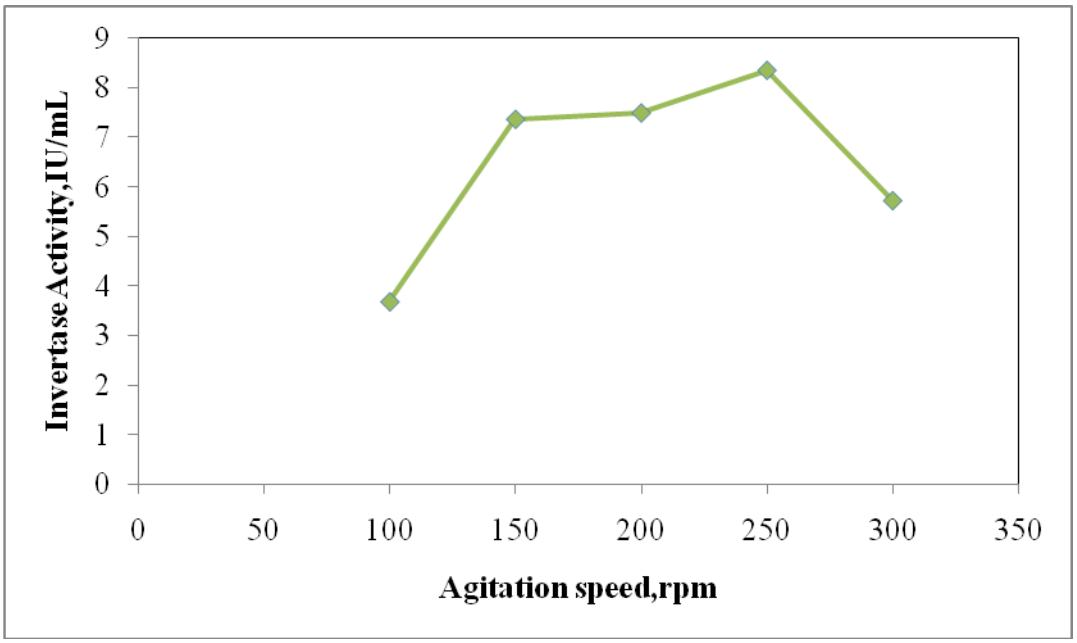

Figure 4.4: Invertase production influence by agitation speed

Agitation speed of the fermentation medium also influence the invertase production. Therefore this study was investigated to examine the effect of agitation speed use in submerged fermentation to the invertase production. The agitation speed of the submerged fermentation was conducted in range 100 rpm to 300 rpm. The collected data were presented in the Appendix F and Figure 4.4 as illustrated above.

Based on the Figure 4.4 above, invertase activity was significantly increased from initial agitation speed use from 100 rpm to 250 rpm which was the maximum

invertase activity yield and begin to decreased in activity from this point to 300 rpm. This shows that this enzyme production was influenced by the agitatioon speed used but higher agitation speed can be a factor that inhibit the production as it may be attributed by the shear stress faced by the biomass pellet (Siti Hatijah, 2010).

Moreover, the similar effect also occur in the Wang *et al.* study in year 2005 in which their study also indicated that due to the shearing effect induced by higher agitation speed had contributed to inactivation of the cells growth and enzyme. It showed that as increasing in the agitation speed there was increasing in cells growth and invertase production until a maximum level of agitation speed before the agitation speed causing the negative effect on the fermentation process.

4.2 Optimization of Invertase Production by Using Response Surface Methodology

Optimization by using one factor at a time method discussed in the previous section was a time concuming and laborous since it is required to determine an optimum conditions and the medium composition to obtain a suitable balance in microbial growth and enzyme yield (Songpim *et al*., 2011). Therefore, a central composite design was used in this study to reduce the time consuming of the experiment used for Response Surface Methdology (RSM) computed by using Design of Experiment Software (DOE).

The fermentation was conducted by using the computed design conditions to determine the maximum invertase production. The three experimental factors computed in the central composite design were sucrose concentration, pH and agitation speed. Each of these variables is varied over five levels which were low axial point ($-\infty$), low factorial point (-1), central point (0), high axial point ($+\infty$) and high factorial point (+1). Table 4.4 shows the list of range and coded level of fermentation process variables. The minimum and maximum values of the parameters were studied based on the value computed in Table 4.5 which shows the design matrix of CCD for extracellular invertase production. Invertase activity (Y) was taken as the response of the design experiment. The full quadratic equation of the response variables for invertase production was obtained as in equation 4.1. The results of the invertase activity were

analyzed using analysis of variance (ANOVA) to evaluate a significant effect if a given term has a significant effect ($p \leq 0.05$). The optimum levels of variables were obtained by graphical and numerical designs using Design expert programmed.

Table 4.1: Experimental range and level coded of process variables

Independent variables	Units	Symbols	Ranges and levels				
			-∞	-1	0	+1	+∞
Substrate concentration	g/L	X_1	3.64	10.00	30.00	50.00	63.64
pH	-	X_2	3.82	4.50	4550	6.50	7.18
Agitation	Rpm	X_3	31.82	100.00	200.00	300.00	368.18

Table 4.2: Table of substrate, pH and agitation speed on invertase activity.

Run	Sucrose concentration in 100mL medium (% w/v)	pH	Agitation Speed (rpm)	Invertase Acitivity (IU/mL)
1	50	4.5	100	17.7677
2	30	5.5	200	26.9583
3	30	5.5	200	26.9583
4	10	6.5	100	11.6261
5	30	5.5	200	26.7383
6	50	4.5	300	21.7314
7	10	4.5	100	14.726
8	30	7.18	200	8.3467
9	30	5.5	31.82	20.8767
10	30	5.5	368.18	19.9074
11	30	5.5	200	26.0827
12	30	5.5	200	26.5395
13	10	6.5	300	6.0851
14	63.64	5.5	200	20.1002
15	10	4.5	300	13.2735
16	30	5.5	200	26.2017
17	50	6.5	100	19.6688
18	50	6.5	300	18.036
19	3.64	5.5	200	13.605
20	30	3.82	200	14.9622

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

Using multiple regressions analysis the following second order equations were found to be fit as models use to produce invertase based on invertase activity that use as response for this analysis (Y) as in equation 4.1 and 4.2 below.

Model equations in terms of coded factors

$$Y= 26.54 + 3.854\ A – 1.70\ B -0.46\ C – 4.34\ A^2 – 5.11\ B^2 – 2.02C^2 + 1.06\ AB + 1.17\ AC -.21BC \quad (4.1)$$

Model equation in terms of actual factors

$$Y = -142.50820 + 0.43445\ A + 55.35729\ B + 0.12539\ C – 0.010839\ A^2 - 5.11164\ B^2 - 2.02243E\text{-}04\ C^2 + 0.053087\ AB + 5.82775\ E\text{-}04\ AC -0.012106\ BC \quad (4.2)$$

Where Y = invertase production (U /mL) or response, A, B, C, A2, B2, C2, AB, AC and BC were the values of independent factors sucrose concentration (g/L), pH and agitation speed (rpm). In order to verify these models it was necessary to conduct an analysis of variance as in Table 4.3.

The data presented in the Table 4.3 described that the regressions for extracellular invertase models obtained in this study was significant (788.48) and those lacked of fits were not significant at $p < 0.0001$ relative to pure error (4.67). The fit of the models was checked by the correlation coefficient, R^2. The R^2 value provided a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value always lied between 0 and 1. The closer the R^2 value to 1.00, the stronger the model was and the better it predicted the response. In this study the value of R^2 for invertase activity was 0.9950. These values showed that 5.80% of the total variables are not explained by the models. The '*Pred* R^2' of, 0.9950 for invertase was reasonable agreement with the '*Adj* R^2' of 0.9904. This indicated a good agreement between the experimental and predicted values

for invertase production. The adjusted R^2 corrected the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2.

Table 4.3: The Anova Result of Invertase Activity

Source of variations	**Sums of squares**	**Degrees of freedom**	**Mean square**	***F*-value**	**Significance (*P* value)**
Regression	788.48	9	87.61	219.63	<0.0001
Residual	3.99	10	0.40		
Lack of fit	*3.29*	5	*0.66*	4.67	0.0580
Pure Error	*0.70*	5	*0.14*		
Total	792.47	19			

Invertase production: R^2 is 0.9950, adjusted R^2 is 0.9904 and predicted R^2 is 0.9646.

The plot of predicted versus actual of invertase activity was shown in the Figure 4.4 for R^2 is equal to 0.9950 below. Based on the plotted graph of predicted versus actual value it was shown that the models obtained was found be excellently fitted.

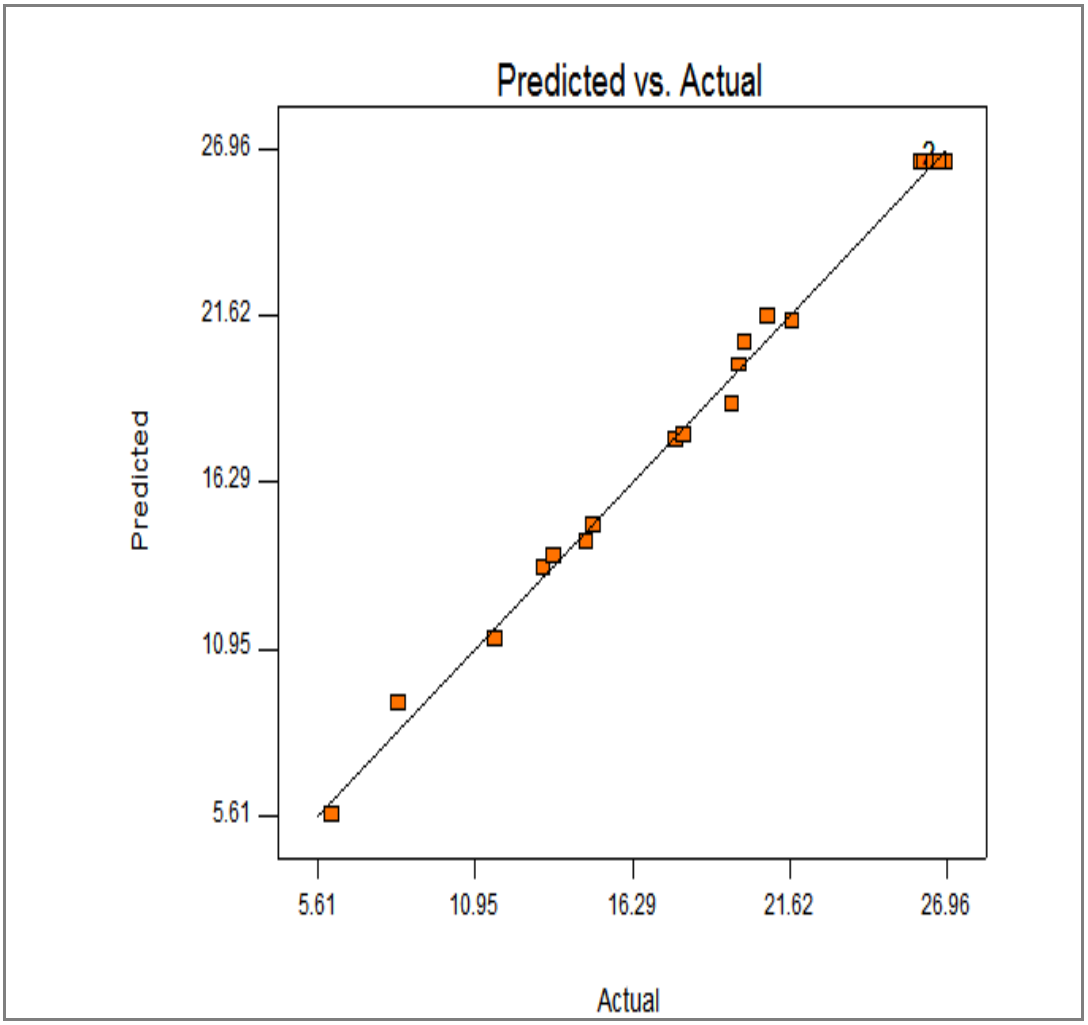

Figure 4.5:Plot of predicted versus actual experimental data for invertase activity.

4.2.1 Interaction of Parameters.

The three-dimensional response surface curves were plotted to study the interaction among different parameters, and to find out the optimum condition for invertase activities. The interactions observed were interaction of substrate concentration with pH, substrate and agitation speed, and pH with agitation speed towards invertase activity.

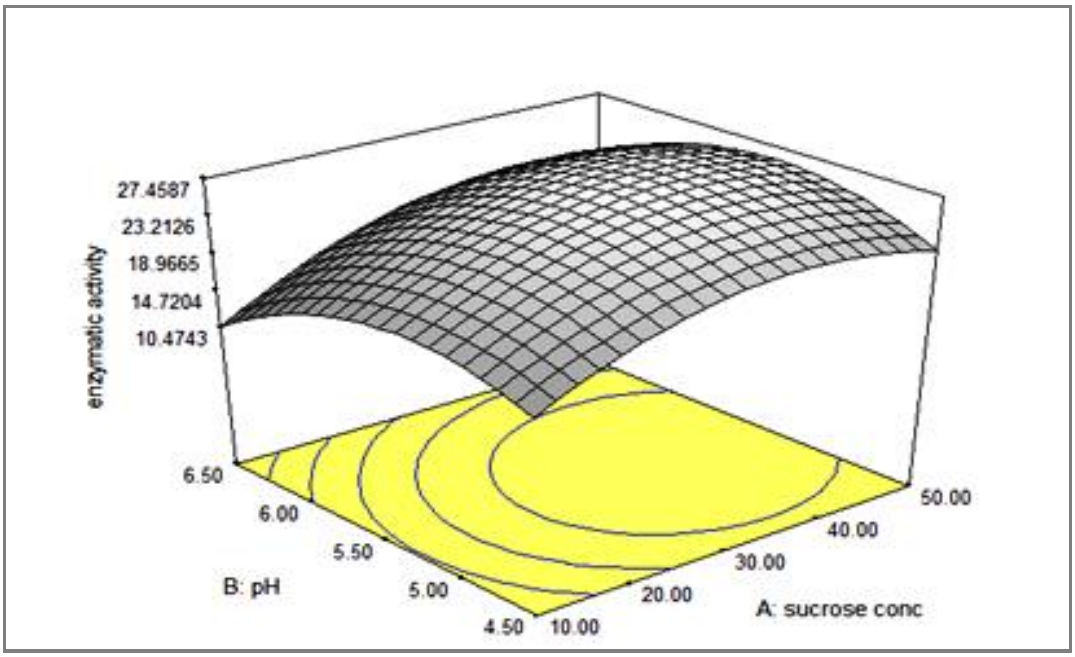

Figure 4.6: Interaction of sucrose concentration and pH towards invertase activity.

Figure 4.6 showed the response of invertase enzyme with respect to sucrose concentration and pH. An increased in pH with sucrose concentration 10 to 30 g/L induced the invertase activity to 26.9583 IU/mL. However, the enzyme activity tends to reduce as the concentration of sucrose increased to 50 g/L though the pH was 5.5. This negative effect may occur due to the inhibition of the sucrose concentration greater than 30 g/L. Furthermore, the increased in the sucrose concentration contribute to the increase of osmotic pressure thus eventually increasing the percentage of cell fractures (Siti Hatijah, 2010). Therefore this study able to indicate the similar effect of interaction of sucrose concentration with the pH compared to previous researchers study.

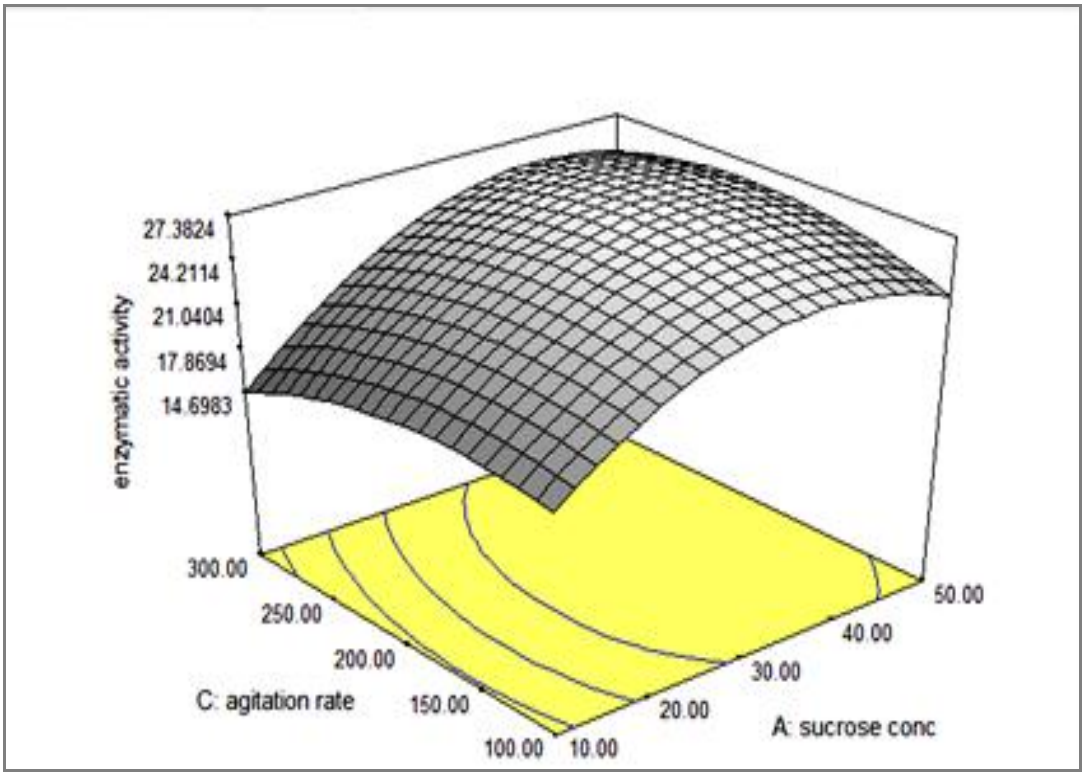

Figure 4.7: Interaction of sucrose concentration and agitation speed towards invertase activity.

The 3D surface for the effect of agitation speed and sucrose concentration was examined and the data collected were presented in the Figure 4.6. It was clearly shown that from the result obtained the invertase production was affected by agitation effect of the medium and sucrose concentration which at higher agitation speed and sucrose concentration the invertase activity was lesser. This shows that enzymes seemed to be affected by higher agitation speed as it may increase shearing effects onto the cells cause the inactivation of cell growth (Wang *et al.*, 2005).

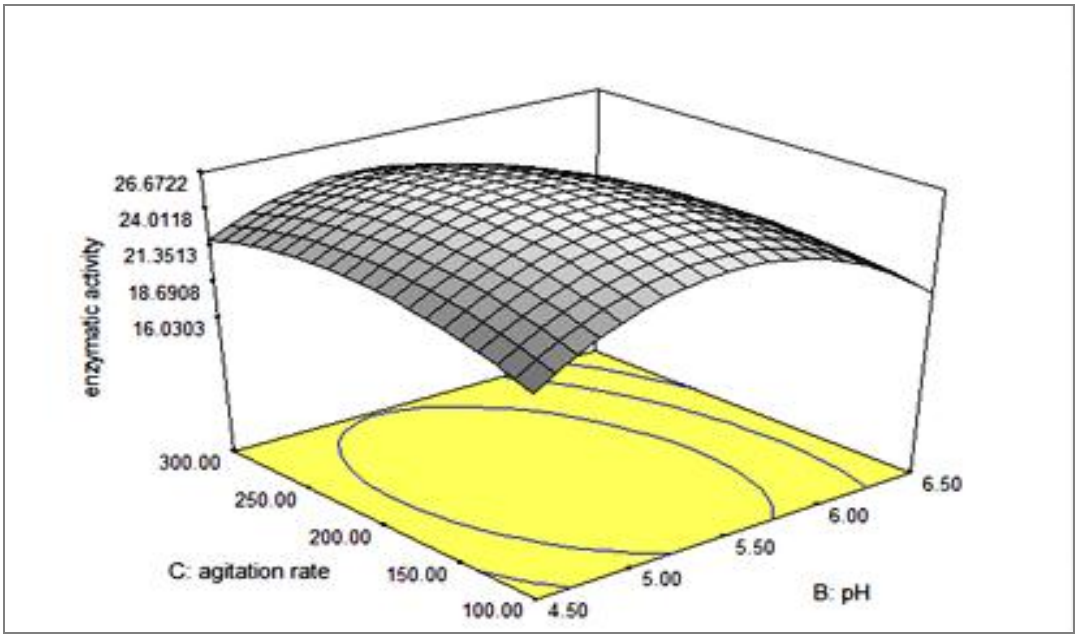

Figure 4.8:Interaction of the pH and agitation speed towards invertase activity.

For the effect of pH and agitation speed it was shown in the Figure 4.7 that indicate that initially the invertase activity slowly increased to reach an optimum level at initial pH and agitation speed use, however it was slowly decreased after the maximum pointwas reach eventually reach the lowest enzyme activity at highest pH and agitation speed. This might occured due to the shearing effect and stability of the medium conditions . According to Wang *et al*.in year 2005 , the shearing effect might be the major causes that influence invertase activity that have been obtained.

4.3 Validation of Models

In order to validate the adequacy of the model Equations (4.1and 4.2), a total of three verification experiments for invertase activity were carried out under various fermentation conditions then the validation data were separately analyzed using the Design Expert ver. 6.0.8 statistical software (Stat-Ease Inc, Minneapolis, MN). The correlation coefficients (R^2) between the experimental and predicted values were 0.9950 and 0.9646 for invertase activity with a significant level of $p<0.001$ The results of analysis indicated that the experimental values were in agreement with the predicted ones, and also suggested that the models of the equations were satisfactory and accurate with error range from 0.005 to 0.10.

Table 4.4: Validation data for optimization process of the invertase activity.

Run				**Invertase Activity, IU/mL**		
	X_1	**X_2**	**X_3**	**Experimental**	**Predicted**	**Error (ε)**
1	30	5.5	200	26.0827	26.54	0.017
2	30	5.5	200	26.9583	26.54	0.016
3	30	5.5	200	26.7383	26.54	0.007

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

As conclusions from this study, the purpose of this study was achieved since the effect of cultivating conditions on biomass production and invertase production for each parameter used to investigate during this study were discovered from range of parameters selected. For the effect of sucrose concentration on the invertase production 30 g/L sucrose concentration shows that the highest value of invertase activity at 8.9132 IU/mL. For the study on the effect of pH towards invertase production, pH 5.5 yields a maximum value of 8.6754 IU/mL invertase production. Agitation speed also one factor that had affected the invertase production in this study At 250 rpm, maximum invertase production at 8.3429 IU/mL has been discovered. This trend has occurred due to the shearing effect of agitation speed on the pellet of fungal that cause it to increase in fractured. For the optimization of the invertase production that have been studied by using these three parameters, the optimum invertase production yield by using 30 g/L sucrose concentration at pH 5.5 and 200 rpm cultivating conditions which was 26.9583 IU/mL with R^2 value of 0.9950 ($P<0.001$).

5.2 Recommendation

Based on the result obtained from this study it was recommended that table sugar was a potential carbon sources that can be used to produce this enzyme since table sugar is a cheap carbon sources can provide a good result in lowering the production cost . It is because the cost of pure sucrose compound is expensive as compared to table sugar.Other than that, since this study was conducted to produce crude invertase enzyme therefore it was recommended to implement another step to produce this enzyme for further study to achive higher yield of enzyme. This can be done by implementing a purification step of the crude enzyme to achieve higher enzyme production with higher quality.

Besides that, the design model which obtained by using central composite design CCD for Response Surface Methodology (RSM) from this study can be used for further study to optimize others parameter that affecting the invertase production in submerged culture using fed batch bioreactor.However, further review on the research that related to determination on effect of cultivating condition to produce this enzyme was strongly recommended due to the time limitation in conducting this study may contribute error in result. Moreover, production of invertase by using bioreactor to obtained high level of invertase production is highly recommend. It is because invertase enzyme provide mulfunction application in food indutries,pharmaceutical industry and etc.

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APPENDIX A

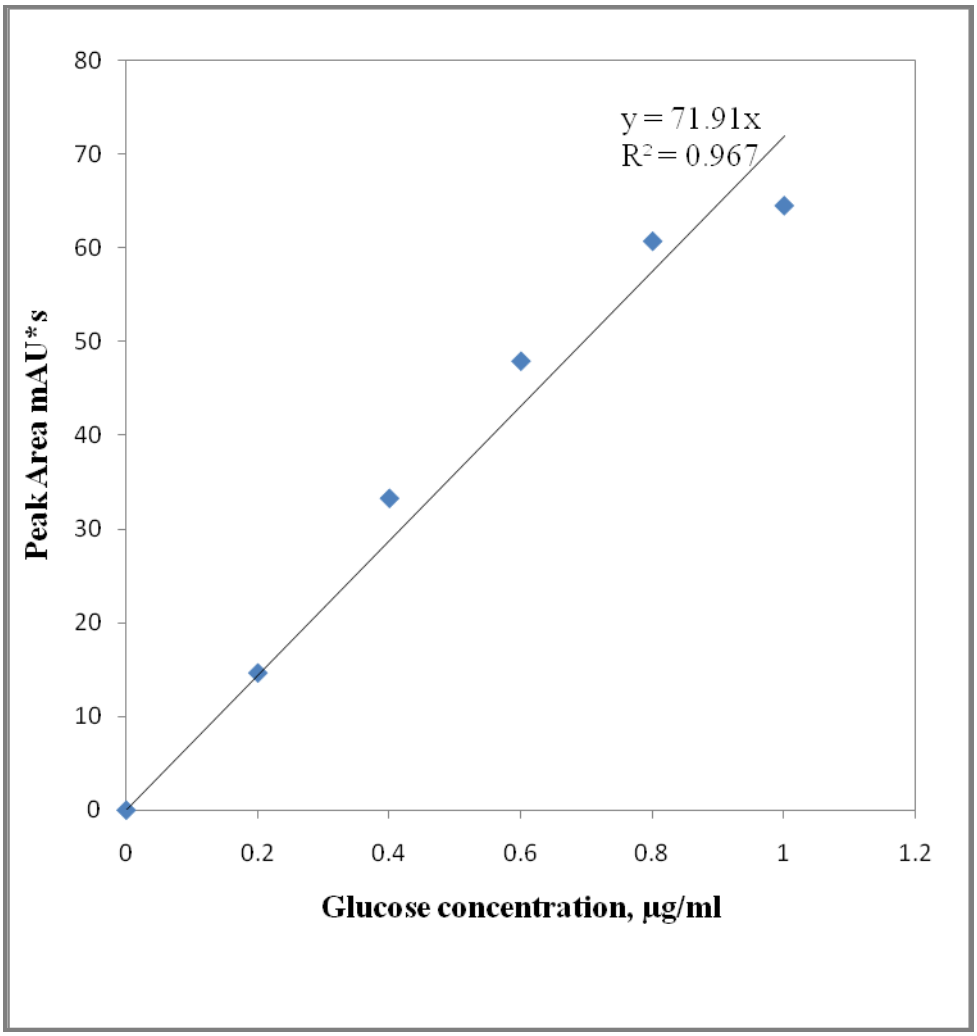
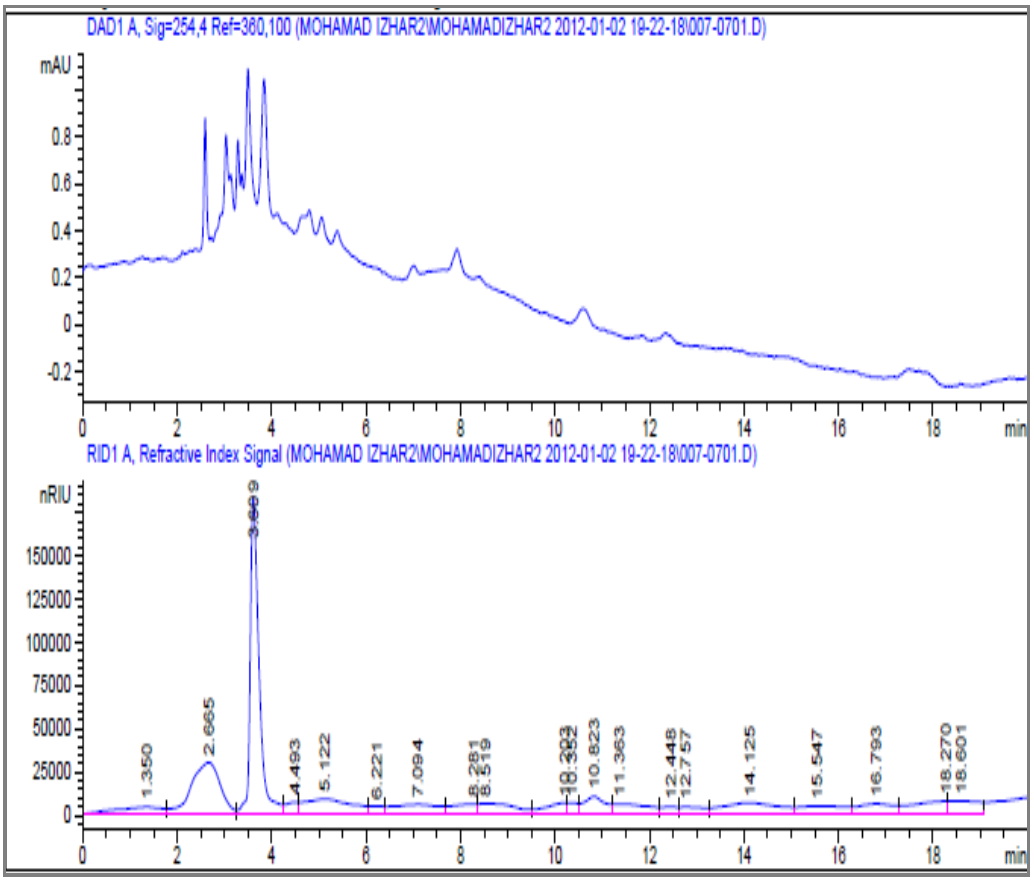


Figure 3.1: Standard Curve of Glucose detection by HPLC

APPENDIX B



HPLC Chromatogram of Sample

APPENDIX C

Table 4.1: Cell dry weight and invertase activity

Time,h	Cell dry weight,g/L	Invertase activity, IU/mL
0.0	1.9125	2.0666
8.0	3.1125	2.2771
16.0	3.3875	2.1565
24.0	3.4725	2.6493
32.0	3.4800	2.9957
40.0	5.1125	3.1586
48.0	4.745	2.6733

APPENDIX D

Table 4.2: Effect of substrate concentration on invertase activity

Substrate Concentration (% g/L)	**Invertase Activity, (IU/mL)**
10.0	4.5605
30.0	8.9132
40.0	6.7001
50.0	6.012

APPENDIX E

Table 4.3: Effect of pH towards invertase activity

pH	Invertase Acitivity, IU/mL
4.5	5.9226
5.0	6.3489
5.5	8.6754
6.0	4.9855
6.5	2.2836

APPENDIX F

Table 4.4: Effect of Agitation Speed on Invertase Activity

Agitation speed, rpm	Invertase Activity IU/mL
100	3.6815
150	7.3564
200	7.4862
250	8.3429
300	5.7183