ENZYME HYDROLYSIS OF OLD NEWSPAPER TO PRODUCE SIMPLE SUGAR FOR FERMENTATION PROCESS

NOOR FAUZANI BINTI AYOP

UNIVERSITY MALAYSIA PAHANG

SUPERVISOR DECLARATION

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

SIGNATURE :

SUPERVISOR NAME : DR. CHUA@YEO GEK KEE

DATE :

STUDENT DECLARATION

"I hereby declare that I have done with wrote this thesis and everything related with undergraduate research project in order to fulfilled the requirement for award of Degree of bachelor of chemical engineering (Biotechnology)"

Signature :

Student name :

Date :

“I dedicate to my beloved parent, friends and especially to my supervisor”

ENZYME HYDROLYSIS OF OLD NEWSPAPER TO PRODUCE SIMPLE SUGAR FOR FERMENTATION PROCESS

NOOR FAUZANI BINTI AYOP

A thesis submitted in partial fulfillment of the
requirements for the award of the degree of
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TABLE OF CONTENT

Acknowledgement vi

Table Of Content vii

List Of Table xi

List of Figures xiii

List of Abbreviations xiv

Abstrak xv

Abstract xvi

CHAPTER 1 – INTRODUCTION

1.1 Background Of Study 1

1.2 Problem Statement 3

1.3 Research Objective 3

1.4 Scope Of Study 4

1.5 Significant Of Proposed Study 4

CHAPTER 2 – LITERATURE REVIEW

2.1 General Overview 5

2.2 Introductions of Lignocellulosic Materials 6

2.3 Pretreatment of Substrate By Non-Ionic Surfactant 8

2.4 Pretreatment of Lignocellulosic Materials 10

2.4.1 Physical Pretreatment 11

2.4.1.1 Mechanical Comminution 11

2.4.1.2 Pyrolysis 12

2.4.2 Physico-Chemical Pretreatment 13

2.4.2.1 Steam Explosion (Autohydrolysis) 13

2.4.2.2 Ammonia Fiber Explosion (AFEX) 14

2.4.2.3 CO_2 Explosion 15
2.4.3 Chemical Pretreatment 15
2.4.3.1 Ozonolysis 15
2.4.3.2 Acid Hydrolysis 16
2.4.3.3 Alkaline Hydrolysis 17
2.4.3.4 Oxidative Delignification 17
2.4.4 Biological Pretreatment 18
2.5 Enzymatic Hydrolysis By Cellulase Enzyme 19
2.6 Overview Of Conversion Cellulose To Glucose By Enzymatic Hydrolysis 20
2.7 Improving Enzymatic Hydrolysis 21

CHAPTER 3 – METHODOLOGY

3. 1 Substrate Preparation 23
3.2 Study The Effect Of Parameters During Enzymatic Hydrolysis For The Glucose Production 24
3.2.1 Surfactant Ratio 24
3.2.2 Enzyme Loading 25
3.2.3 pH Buffer 25
3.2.4 Incubation Temperature 26
3.2.5 Agitation Speed 26
3.2.6 Incubation Time 27
3.3 Glucose Determination 27

CHAPTER 4 – RESULTS AND DISCUSSIONS

4.1 Introduction 28
4.2 Effect Of Surfactant Ratio 29
4.3 Effect Of Enzyme Loading 31
4.4 Effect Of pH Buffer 33
4.5 Effect Of Incubation Temperature 34
4.6 Effect Of Agitation Speed 35
4.7 Effect Of Incubation Time 37

CHAPTER 5 – CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion 39

5.2 Recommendation 40

REFERENCES 41

APPENDICES

A – Citrate Buffer 44

B – Glucose Determination 44

C – DNS Method 45

D – General Procedure 46

E - Results 47

LIST OF TABLE

		Page
Table E.1.1	Optical density of glucose production from surfactant ratio at 540nm	39
Table E.1.2	Glucose standard curve for surfactant ratio	39
Table E.1.3	Glucose concentration of samples surfactant ratio obtained from glucose standard curve	40
Table E.2.1	Optical density of glucose production from enzyme loading at 540nm	41
Table E.2.2	Glucose standard curve for enzyme loading	41
Table E.2.3	Glucose concentration of samples enzyme loading obtained from glucose standard curve	42
Table E.3.1	Optical density of glucose production from pH buffer at 540nm	43
Table E.3.2	Glucose standard curve for pH buffer	43
Table E.3.3	Glucose concentration of samples enzyme loading obtained from glucose standard curve	45
Table E.4.1	Optical density of glucose production from incubation temperature at 540nm	45
Table E.4.2	Glucose standard curve for incubation temperature	46
Table E.4.3	Glucose concentration of samples incubation temperature obtained from glucose standard curve	47
Table E.5.1	Optical density of glucose production from agitation speed at 540nm	47
Table E.5.2	Glucose standard curve for agitation speed	48
Table E.5.3	Glucose concentration of samples agitation speed obtained	49

	from glucose standard curve	
Table E.6.1	Optical density of glucose production from incubation time at 540nm	49
Table E.6.2	Glucose standard curve for incubation time	50
Table E.6.3	Glucose concentration of samples incubation time obtained from glucose standard curve.	51

LIST OF FIGURE

		Page
Figure 2.1	Schematic of the role pretreatment in the conversion cellulose to glucose	9
Figure 2.2	Overview of conversion cellulose to glucose by enzymatic hydrolysis	16
Figure 4.1	Effect of surfactant ration (TX-21: TWEEN 80) on glucose production	24
Figure 4.2	Effect of enzyme loading on glucose production	25
Figure 4.3	Effect of buffer's pH on the glucose production	26
Figure 4.4	Effect of incubation temperature on the glucose production	28
Figure 4.5	Effect of agitation speed on the glucose production	29
Figure 4.6	Effect of incubation time on the glucose production	31
Figure E.1	Glucose standard curve that be used for determine glucose concentration from samples of surfactant ratios	40
Figure E.2	Glucose standard curve that be used for determine glucose concentration from samples of enzyme loading	42
Figure E.3	Glucose standard curve that be used for determine glucose concentration from samples of buffer's pH	44
Figure E.4	Glucose standard curve that be used for determine glucose concentration from samples of incubation temperature	46
Figure E.5	Glucose standard curve that be used for determine glucose concentration from samples of agitation speed	48
Figure E.6	Glucose standard curve that be used for determine glucose concentration from samples of incubation time.	50

LIST OF ABBREVIATIONS

AFEX	Ammonia fiber explosion
DNS	3,5-dinitrosalicyclic acid
NP	Nonylphenol
OFAT	One-factor-at-a time
TW	Tween
UV-Vis	Ultraviolet-visible

ENZYME HYDROLYSIS OF OLD NEWSPAPER TO PRODUCE SIMPLE SUGAR FOR FERMENTATION PROCESS

ABSTRAK

Penghasilan penapian gula dari bahan lignoselulosa telah dikaji oleh ramai penyelidik. Pada masa kini, banyaknya terhasil bahan buangan lignoselulosa disekeliling dunia termasuk juga di Malaysia. Surat khabar adalah sumber bahan lignoselulosa kerana surat khabar itu sendiri diperbuat daripada kayu lembut atau pulpa kayu yang mengandungi bahan lignoselulosik iaitu selulosa, separa-selulosa dan lignin. Selulosa adalah komponen terbesar pembahagiannya dengan 45 – 50 %. Selulosa juga boleh ditukarkan kepada gula mudah iaitu glukosa. Hidrolisis enzim lebih digalakkan untuk proses penukaran kerana lebih jimat dan kadar penukaran yang lebih banyak. Dalam kajian ini, enzim cellulase dari Novozyme telah digunakan kepada hidrolisis surat khabar untuk penukaran glukosa. Dengan mengunakan satu-faktor-pada- satu masa (OFAT), enam pembolehubah telah dikaji. Pembolehubahnya adalah nibah surfaktan, penambahan enzim, penampan pH, suhu pengeraman, kelajuan pergolakkan dan waktu pengeraman. Didalam ekperimen ini, penghasilan glukosa telah diukur mengunakan kaedah DNS. Keputusan yang dapat telah menunjukkan pada nisbah surfaktan 0.2:0.8 untuk TX-21:TWEEN-80. 1.0 ml enzim cellulase pada pH 6.0 apabila dimasukkan bersama 0.2 g surat khabar pada 50 ^{o}C dan pergolakan 200 rpm untuk 96 jam adalah sesuai untuk reaksi enzim. Pembolehubah-pembolehubah yang telah dapat memberikan penghasilan glukosa yang tertinggi iaitu pada 0.73 g/L.

ENZYME HYDROLYSIS OF OLD NEWSPAPER TO PRODUCE SIMPLE SUGAR FOR FERMENTATION PROCESS

ABSTRACT

Production of fermentable sugar from lignocellulosic material was attempted by many researchers. Nowadays, this is because of plenty lignocellulosic biomass waste available around the world including Malaysia. Newspaper is a type of lignocellulosic sources because newspaper was made from softwood that contains cellulose, hemicelluloses and lignin. Cellulose component in newspaper is the major component with 45-50 %. The celluloses were able to be converted into simple sugar, which is glucose. Enzymatic hydrolysis is preferred for the conversion because less cost and high conversion. In this study, cellulase enzyme from Novozyme was used to hydrolyze newspaper for glucose production. By applying one-factor-at-time (OFAT), six parameters were being studied. They are surfactant ratio, enzyme loading, buffer pH, incubation temperature, agitation speed and incubation time. In all experiments, glucose production was monitored by DNS method. The result obtained show that at surfactant ratio 0.2:0.8 of TX-21: TWEEN-80, 1.0 ml of cellulase enzyme, at pH 6.0 when mixed with 0.2 g substrate at 50 ^{o}C and agitated at 200 rpm for 96 h is suitable for enzyme reaction. Those optimum parameters obtained gave the higher glucose production at 0.73 ± 0.02 g/L.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Lignocellulose biomass is the most abundant raw material waste on the earth. Abundant newspaper waste was generated every year from all over the world due to high production rate of newspapers. This has become one of the severe environmental issues.

Lignocellulosic biomass is favorable for its highly abundance and high energy potential. Lignocellulosic materials consist of three major component, they are cellulose, hemicelluloses and lignin. The bioconversion of lignocellulosic materials is now a subject of intensive research as a contribution to the development of large scale conversion

process beneficial to mankind (Kumakura, 1997). In newspaper, cellulose comprises the largest fraction of the biomass ranging from 30% to 50% (MacLellan, 2010) that can be degrading to smaller monomer of fermentable sugar such as glucose. In fermentation process, a carbon source is the most important feed to produce valuable product. In this case, glucose is widely use in fermentation process for such purpose.

Lignocellulosic materials are complex carbon source that need to go through pretreatment process. Only enzyme can fully degrade the newspaper to fermentable sugar, which is called enzymatic hydrolysis. Enzymatic hydrolysis is a process of degrading biomass sample using enzyme under suitable condition to produce fermentable sugar. In pretreatment of lignocellulosic material, enzymatic hydrolysis is preferred because of the higher conversion yield, less corrosive, less toxic condition as compared to others pretreatment method such as physical pretreatment, physico-chemical pretreatment, chemical pretreatment and biological pretreatment (Sun, 2002). The enzyme that can degrade cellulose to glucose is cellulase enzyme.

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulose hydrolysis is accomplished with the aid of cellulase enzyme complex which is made up of three classes of enzymes namely exoglucanase, endoglucanase and β-glucosidase (Beguin, 1990). Cellulase enzyme is a group of enzyme that acting to break the polymer chain of cellulose to monomer chain of glucose (MacLellan, 2010). Because of too many factors need to considered, all the parameters during the process have to be examined in order to understand the importance and effect of the parameters for optimizing the production of glucose from newspaper.

1.2 Problem Statement

To obtain the higher fermentable glucose production from old newspaper using cellulase enzyme by enzymatic hydrolysis, recognizing the important parameters that affect enzymatic hydrolysis are very important. Furthermore, Novozyme Cellulase enzyme complex, is a newly developed commercial enzyme, there is no study has been done in its application on hydrolyzing newspaper to glucose production. Therefore, investigation on the parameters that affect enzymatic hydrolysis is necessary.

1.3 Research Objective

To study the effect of various operating parameters during enzymatic hydrolysis of newspapers to fermentable glucose by cellulose.

1.4 Scope Of Study

The scope of study was to determine the effect of all operating parameters that would influence enzymatic hydrolysis in producing high amount of glucose i.e. surfactant ratio, pH buffer, enzyme loading, agitation speed, incubation temperature and incubation time.

1.5 Significant Of Proposed Study

Current world trend has moved towards paperless concept. However, in certain countries and certain tasks, such as legal document, newspaper and magazine, still need to use paper especially in Malaysia. Popularity of using paper has generated lots of paper waste per annum. Then, these papers were collected and recycled to produce recycled paper, but after several cycle, the paper quality decrease and this give disadvantaged to the biomass content because the conversion will be lower. Instead of generating tons of waste every year, this biomass can be used to convert to more valuable product such as glucose or as feedstock to generate renewable energy that can replace current petroleum based liquids fuels or others bioproducts.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Overview

Production of newspaper per annum was abundance in Malaysia because newspaper was produced everyday. Besides that, the used newspaper has been recycled as recycle paper, but recycled paper is low in quality and if recycle for several time, it will become useless product. Therefore, in order to generate less solid waste, newspaper should be transformed to some other valuable products. Through this study, the old newspaper was used to convert the cellulose component in it into the sugar, where this sugar was able to produce another more valuable product such as biofuel. Newspaper is

made from softwood that contained lignocellulosic materials, where the major component is cellulose. Enzymatic hydrolysis of newspaper by cellulase enzyme is preferred because of its ability to convert the cellulose into high quantity of fermentable sugar (Chu and Feng, 2012), which is a more efficient pretreatment method compared to others. To improve and obtain the optimum value for production of glucose, all factors that affecting the process will be examined starting from initial pretreatment until enzymatic hydrolysis of newspaper.

2.2 Introductions of Lignocellulosic Materials

Sun and Cheng (2002) stated that cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. According to Rao (2009), it is composed of about 30-50 % cellulose, 20-35 % of hemicelluloses and 10-15 % lignin. This linear polymer consists of D-glucose subunits linked to each other by β-(1,4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils (Zhang and Lynd, 2004). Hemicelluloses and lignin, on the other hand, are found cover the microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the β-(1,4)-glycosidic linkages (Gusakov *et. al.*, 2006).

Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form. The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. These monosaccharides include pentoses, hexoses, and uronic acids. The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1, 4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds. Also, hemicelluloses can have some degree of acetylation. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolyzable. These polymers do not aggregate, even when they cocrystallize with cellulose chains.

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack. Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl−aryl, alkyl−alkyl, and aryl−aryl ether bonds link these phenolic monomers together. In general, herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents (Kumar *et. al.*, 2009).

2.3 Pretreatment of Substrate by Non-Ionic Surfactant

To improve the accessibility of the substrate, which is old newspaper, it is required to pretreat it before undergo to sugar production. As stated by Kim *et. al.* (2007), cellulose is the major component of newspaper that is able to convert into fermentable sugars by enzymatic hydrolysis. Enhancement of cellulose hydrolysis by adding surfactants to the hydrolysis mixture has been reported by (Chandra *et. al.*, 2008). Eriksson *et. al.* (2002) was compared amorphous cellulose with different types of crystalline celluloses (Avicel, tissue paper and reclaimed paper). They showed that the higher the crystallinity of the substrate, the more positive was the effect of the added surfactant.

However, inks and certain additives used in paper production can hinder enzyme access to the substrate. Thus, an effective pretreatment is an essential step to increase the enzymatic digestibility of waste paper and reducing quantity of enzyme. According to Kumar *et. al.* (2009) and also Sun and Cheng (2002), the composition of lignocellulosic material of newspaper is cellulose 40-55 %, hemicelluloses 25-40 % and lignin 18-30 %. Kim and Feng (2007) believe that surfactants can help to remove the ink and other components that physically interfere with enzymatic hydrolysis.

Studies of pretreatments that were based on surfactants alone are very limited. Furthermore, the addition of a surfactant to the enzymatic hydrolysis stage of lignocelluloses increases the conversion of cellulose to glucose. When newspaper was pretreated to increase its enzymatic digestibility, the pretreatments that based on a surfactant alone showed higher digestibility than the pretreatments that based on Tween-

series surfactants, ammonia, and/or hydrogen peroxide. According to Hwang *et. al.* (2008), TWEEN-80 has been proposed because TWEEN 80 able to increased microbial growth, cellulolytic enzyme activity and enzyme binding on substrate. Therefore, TWEEN-80 may improve fiber digestion and made enzymatic degradation of cellulose easier. Moreover, non-ionic surfactant decreases the absorption of enzyme to substrate, which may aid to maintain enzymatic reaction. According to Kim *et. al.* (2007) in their preliminary experiments, NP-20 showed slightly better performance than NP-5 and NP-10, and therefore, NP-20 was selected for further experimentation together with TW-80 on pretreatment performance. The digestibility of the NP-20- pretreated substrate was approximately 10 % higher than that of TW-80-pretreated substrate. Significant surfactant effect with addition NP-20 and TWEEN-80 were give high digestibility α-cellulose of newspaper compares to small surfactant,

In addition, the surfactant TX-21 that has similar characteristic such as hydroxyl value at 44.54 mgKOH/g, the moisture content at 1.0 % with pH 5.0 – 7.0 with the NP-20 that can give similar behave if mix with TWEEN-80. Therefore, the reason of combination TWEEN-80 with TX-21 is to complete the process where TWEEN-80 was de-inking the newspaper and TX-21 remove all the impurities including surfactants during substrate washing with deionized water (Kim *et. al.*, 2007).

2.4 Pretreatment of Lignocellulosic Materials

Pretreatment of lignocellulosic materials can convert cellulose to fermentable glucose. Glucose acts as carbon source that is an important nutrient for microorganism to produce valuable product such as biofuels. The aim of pretreatment process is to remove lignin and hemicelluloses, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials. Pretreatment must have the following requirement. First is proving the formation of sugar or the ability to subsequently form sugar by hydrolysis. Second is to avoid the degradation or loss of carbohydrate. Third is avoid the formation of byproducts that are inhibitory to the subsequently hydrolysis and fermentation process and last is be cost-effective. Pretreatment methods can be divided into different categories which are physical (milling and grinding), physicochemical (steam pretreatment, hydrothermolysis and wet oxidation), chemical (alkali, dilute acid, oxidizing agent and organic solvent), biological or electrical (Kumar *et. al.*, 2009).

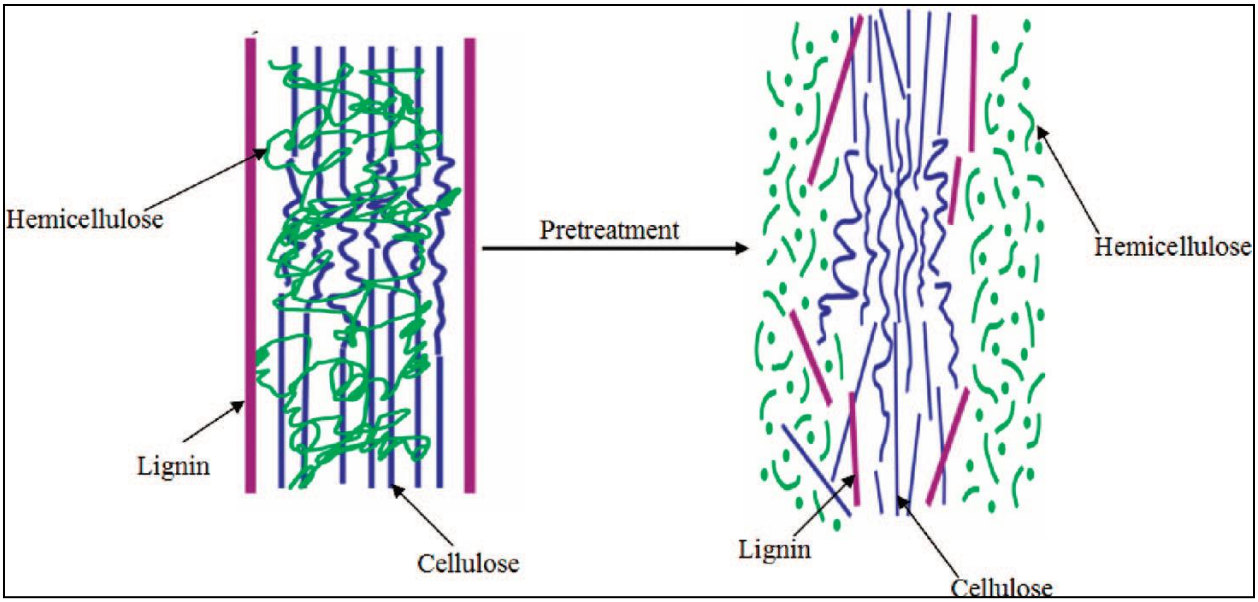

Figure 2.1 An illustration of pretreatment lignocellulosic materials by surfactant. (Source: Kumar *et. al*, 2009)

2.4.1 Physical Pretreatment

2.4.1.1 Mechanical Comminution

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling. The power

requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.1.2 Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300 ^{o}C, cellulose rapidly decomposes to produce gaseous products and residual char. The decomposition is much slower and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1 N H_2SO_4, 97 ^{o}C, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80–85 % conversion of cellulose to reducing sugars with more than 50 % glucose. The process can be enhanced with the presence of oxygen. When zinc chloride or sodium carbonate is added as a catalyst, the decomposition of pure cellulose can occur at a lower temperature (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.2 Physico-Chemical Pretreatment

2.4.2.1 Steam Explosion (Autohydrolysis)

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials. In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160–260 ^{o}C for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. The factors that affect steam explosion pretreatment are residence time, temperature, chip size and moisture content. Addition of H_2SO_4 or CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose. Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes. Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicelluloses. The water wash decreases the overall

saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.2.2 Ammonia Fiber Explosion (AFEX)

AFEX is another type of physicochemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg ammonia/kg dry biomass, temperature 90 ^{o}C, and residence time 30 min. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. The composition of the materials after AFEX pretreatment was essentially the same as the original materials. However, the AFEX process was not very effective for the biomass with high lignin content such as newspaper (18–30% lignin) and aspen chips (25 % lignin). Hydrolysis yield of AFEX-pretreated newspaper and aspen chips was reported as only 40 % and below 50 %, respectively. To reduce the cost and protect the environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with a temperature up to 200 ^{o}C was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.2.3 CO_2 Explosion

Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Compared CO_2 explosion to steam with ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse, and repulping waste of recycled paper, then found that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds which could occur in steam explosion. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.3 Chemical Pretreatment

2.4.3.1. Ozonolysis

Ozone can be used to degrade lignin and hemicelluloses in many lignocellulosic materials. The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. Enzymatic hydrolysis yield increased from 0 % to 57 % as the percentage of lignin decreased from 29 % to 8 % after ozonolysis pretreatment of poplar sawdust. Ozonolysis pretreatment has the following

advantages, first is it effectively removes lignin. Second it does not produce toxic residues for the downstream processes. Lastly, the reactions are carried out at room temperature and pressure. However, a large amount of ozone is required, making the process expensive. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.3.2 Acid Hydrolysis

Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible.

Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis. At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis. Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physicochemical pretreatment processes such as steam explosion or AFEX. A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.3.3 Alkaline Hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials. The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, the porosity of the lignocellulosic materials increases with the removal of the crosslinks. Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. The digestibility of NaOH-treated hardwood increased from 14 % to 55 % with the decrease of lignin content from 24–55 % to 20 %. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26 %. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.3.4 Oxidative Delignification

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2. The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50 % lignin and most hemicelluloses were solubilized by 2 % H_2O_2 at 30 ^{o}C within 8 h, and 95 % efficiency of glucose production from cellulose was achieved in the subsequent saccharification by

cellulase at 45 ^{o}C for 24 h . Used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170 ^{o}C, 5–10 min), and achieved 85 % conversion yield of cellulose to glucose. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.4.4. Biological Pretreatment

In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials. Studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordida* 37 and *Pycnoporus cinnabarinus* 115 in four weeks. In order to prevent the loss of cellulose, a cellulase-less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips and the delignification of Bermuda grass by white-rot fungi. The biodegradation of Bermuda grass stems was improved by 29–32% using *Ceriporiopsis subvermispora* and 63–77% using *Cyathus stercoreus* after 6 weeks. The white-rot fungus *P. chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese- dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation. Both enzymes have been found in the

extracellular filtrates of many white-rot fungi for the degradation of wood cell walls. (Sun and Cheng, 2002; Kumar *et. al.*, 2009)

2.5 Enzymatic Hydrolysis by Cellulase Enzyme

This study applies enzymatic hydrolysis using cellulase enzymes which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is lower compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 50 ^{o}C) and does not have a corrosion problem (Mussatto *et. al.*, 2008). The bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials.

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process. First is endoglucanase which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends. Second is exoglucanase or cellobiohydrolase which degrades the molecule further by removing cellobiose units from the free chain-ends and third is b-glucosidase which hydrolyzes cellobiose to produce glucose. In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, b-xylosidase, galactomannanase and glucomannanase. During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to valuable products. This is also supported by MacLellen (2010), where enzymatic hydrolysis is preferred because

of the higher conversion yields and less corrosive, less toxic conditions compared to an acid hydrolysis.

2.6 Overview of Conversion of Cellulose to Glucose by Enzymatic Hydrolysis

Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulases are widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms. Cellulases play a significant role in the enzymatic process by catalyzing the hydrolysis of cellulose to soluble, fermentable sugars. As stated above, there are three major type of cellulase enzymatic activities that believed to be involved in cellulose hydrolysis based on their structural properties, which are endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases, exoglucanases, including 1,4-β-D glucan glucanohydrolases and 1,4-β-D-glucan cellobiohydrolases, and β-glucosidases or β-glucoside glucohydrolases (Lynd *et al*., 2002).

Figure 2.2 shows mechanism of conversion cellulose to glucose. It begins with endoglucanases cut at random internal sites of the amorphous cellulose polysaccharide chain, generating oligosaccharides of various lengths with new chain ends, which is shortest than original. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose.

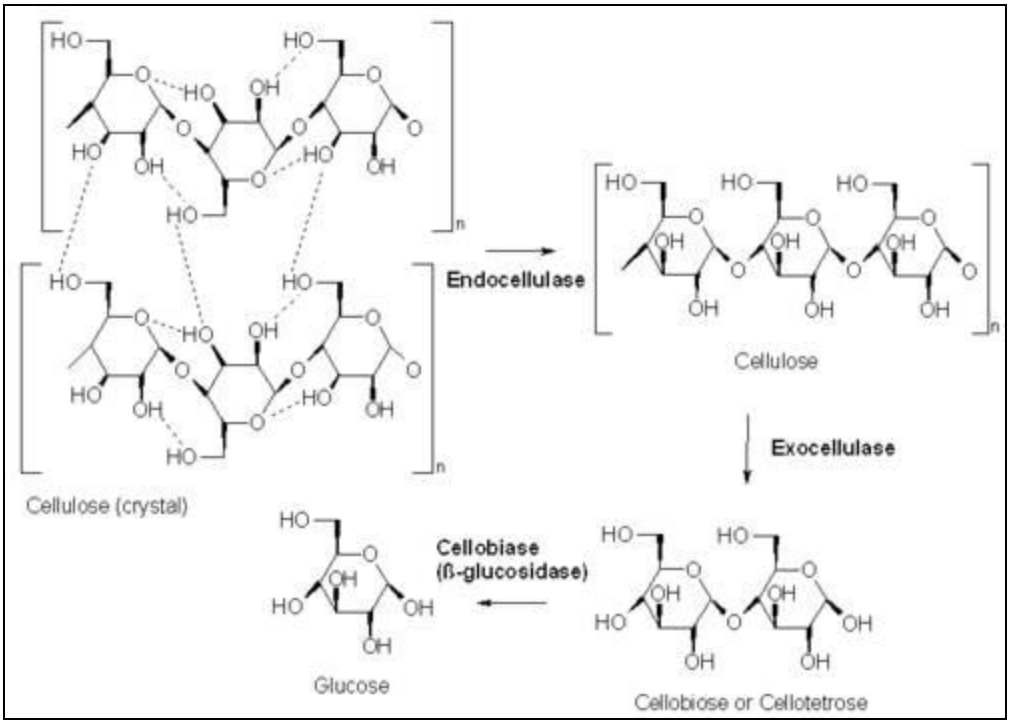

Figure 2.2 Overview of conversion of cellulose to glucose by enzymatic hydrolysis (Source: Qing, 2010)

2.7 Improving Enzymatic Hydrolysis

In order to determine the higher glucose production, the overall processes need to study so that the optimum conditions were being achieved. It is apparent that different characteristics within the lignocellulosic substrates can limit hydrolysis by the cellulose system. However, the action of cellulases also alters the natural characteristics of lignocellolosic substrates as hydrolysis proceeds. To improve the pretreatment enzymatic hydrolysis, it is most important to study the parameter involved.

The factors that affect the enzymatic hydrolysis include substrates concentration, cellulase activity, and reaction conditions (temperature, pH, etc). In this study, the parameters that are going to study and optimize include surfactant ratio, agitation speed for substrate pretreatment, buffer's pH, enzyme loading, incubation temperature and hydrolysis time for enzymatic hydrolysis.

CHAPTER THREE

METHODOLOGY

3.1 Substrate Preparation

The old newspaper was cut into 2 cm x 2 cm size and stored dry in the zipper bag. During the experiment, 10g of these newspapers were used for each flask in all the experiments.

3.2 Study the Effect of Parameters during Enzymatic Hydrolysis for the Glucose Production

3.2.1 Surfactant Ratio

10 g of dried old newspaper was added into 200 mL of deionized water in a 500 mL beaker. After that 1 mL of surfactant TX-21 and Tween-80 was prepared at a ratio of 4:1. The surfactant mixture was stirred with magnetic stirrer for 1 hour. After 1 hour, the substrate was washed with deionized water to remove the surfactant and other material, and then the substrate was dried in the oven at 60 ^{o}C for 24 hours. The dried substrate was stored in zipper bag at room temperature. The above pretreatment steps were repeated for TX-21 and Tween-80 ratio of 0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2, 1.0:0.0, 0.0:1.0 and 0:0.

Sodium citrate buffer at pH 4.8 was added into each test tube at a volume of 9.5 ml. 0.5 ml cellulase enzyme (Novozyme, CVN04025) was then added together with the substrate. The same mixture was prepared for each substrate that has different surfactant ratio. The test tube was covered and incubated at 50^{o}C, 200rpm for 24 hours in the incubator shaker (Kuhner). After 24 hours, the sample was heated to 90 ^{o}C for 10 minutes to inactivate the enzyme. Then, using a bench top centrifuge (Eppendorf centrifuge 5810 R), the sample was separated from the substrate at 10,000rpm for 5 minutes and the

supernatant was collected. The glucose concentration in the sample was analyzed by using DNS method.

3.2.2 Enzyme Loading

This study used the dried substrate from the optimum surfactant ratio of TX-21 and Tween-80 obtained from section 3.2.1, that is 0.2:0.8. The procedures for enzymatic hydrolysis is similar to that has been described in the section 3.2.1, but the volume for cellulase enzyme (Novozyme) utilized was different. The volume of enzyme study was 0.0 ml, 0.2 ml, 0.5 ml, 0.8 ml, 1.0 ml, 1.2 ml and 1.5 ml in 10 ml of total volume.

3.2.3 pH Value

The optimum surfactant ratio of TX-21 and TWEEN-80 and optimum enzyme loading from previous study were used in this study. The pretreatment step was identical with the one described in the section 3.2.1. The pH value for sodium citrate buffer was varied at pH 3, 4, 5, 6, 7, and 8. All other procedures were similar to those described in the section 3.2.1 by using the optimum enzyme loading from section 3.2.2.

3.2.4 Incubation Temperature

In this study, all optimum values obtained from section 3.2.1, 3.2.2 and 3.2.3 will be employed. Different temperature was used for this section where the temperature was set at 40^{o}C, 50^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C. All other settings were not changed.

3.2.5 Agitation Speed

All the optimum conditions obtained from section 3.2.1 until 3.2.4 were employed in studying this parameter. The agitation speed was changed to 100rpm, 200rpm, 300rpm and 400rpm. All other procedures were exactly similar with that described in the section 3.2.1. A static run was also performed for comparison.

3.2.6 Incubation Time

In this section, all the optimum parameters that were obtained from section 3.2.1 until 3.2.5 were used. However, the incubation time was varied at 24 h, 48 h, 72 h, 96 h and 120 h. The procedures of enzymatic hydrolysis were identical to the above.

3.3 Glucose Determination

To determine the glucose concentration, DNS method was used. Glucose standard curve was prepared at the concentration 0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L. 10mL of blank solution was also prepared, which contained sodium citrate buffer and deionized water. In the assay, 3ml of DNS reagent was added into 3ml of sample (glucose standard or experimental samples) in the test tube. The test tube was soaked into water bath at 90^{o}C for 10 minutes. The samples was then cool down at room temperature, 1 ml of potassium sodium tartrate was added to the cooled samples and mixed properly. The optical density of glucose was then measured using UV-Vis spectrophotometer (Hitachi U-1800) at 540nm against blank solution. A standard curve of glucose was plotted and this was used to determine the concentration of glucose in the samples.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Introduction

Production of glucose from old newspaper was obtained by enzymatic hydrolysis method. This chapter presented the result of various effects that affecting the glucose production via enzymatic hydrolysis. The parameters studied included surfactant ratio, enzyme loading, buffer's pH, incubation temperature, agitation speed and incubation time. All effects on the glucose production from the changes of the parameters would be discussed.

4.2 Effect of Surfactant ratio

Newspaper was typically pretreated prior to enzyme hydrolysis to increase its enzymatic digestibility. Tween-series surfactant, ammonia and hydrogen peroxide are some of the chemicals that were used for this purpose. In this study, TX-21 and TW-80 were selected to investigate the effect of surfactant type on pretreatment of newspaper. TX-21 and TW-80 were mixed at various ratio as shown in Table 4.1 at appendix during the pretreatment stage and the pretreated newspapers were then hydrolyzed following the procedure describe in section 3.2.1.

Figure 4.1 shows the glucose concentration obtained via enzymatic hydrolysis of newspaper using different surfactant ratio during pretreatment. It can be seen that surfactant ratio of 0.2:0.8 (TX-21: TW-80) gave the highest value of glucose production. However, the increment was only slightly higher than other mixing ratio of surfactants. This is because based on the error bar values, the surfactant ratio of 0.2:0.8 (TX-21: TW-80) gave very similar glucose production with that using surfactant ratio of 0.8:0.2, 0.6:0.4 and 1.0:0.0 (TX-21: TW-80). Enzymatic hydrolysis of the newspaper with no surfactant added, the glucose production was the lowest which at 0.27 g/L. However, Duff *et. al.* (1995) reported that TWEEN-80 only marginally increased sugar production when disposal sludge from de-inking mill was hydrolyzed with cellulase. According to them, the surfactant might leave in the pretreated substrate after washing, which may affect the hydrolysis.

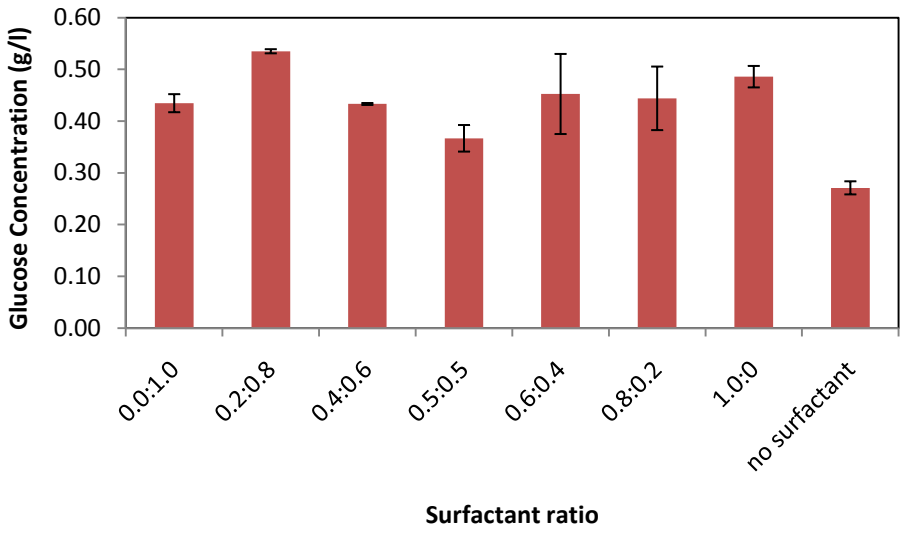

Figure 4.1 Effect of surfactant ration (TX-21: TWEEN 80) on glucose production

In this study, glucose production was increased from 0.26 g/L in enzymatic hydrolysis with no surfactant increase to 0.54 g/L using 0.2:0.8 (TX-21: TW-80), a near 2-fold increment. Therefore, the observation was similar to that reported by Wu (1998). This may be due to a mixture of surfactants (TX-21 and TW-80) were being used instead of single type surfactant as that reported by Duff *et. al.* (1995). Cellulase activity will decrease during hydrolysis. By addition of surfactant during hydrolysis, it is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulase on cellulose (Sun and Cheng, 2002). Non-ionic surfactants are believed to be suitable surfactant for enhancing the cellulose hydrolysis. In this study, both TX-21 and TW-80 were non-ionic surfactants. Thus their mixture may augment the effect. From Figure 4.1, 0.2:0.8 (TX-21: TWEEN-80) is the most optimum ratio to be used in order to obtain high glucose concentration.

4.3 Enzyme Loading

Enzyme loading is defined as the amount of enzyme used to catalyze a unit mass of substrate. This parameter was investigated in order to know the optimum amount of enzyme should be used to obtain the maximum glucose in shorter time. This was studied by using surfactant ratio of 0.2:0.8 (TX-21: TW-80) from section 4.2 that gave the highest glucose production according to different amount of enzyme ranging from 0.2 ml until 1.5 ml per 10g of pre-treated substrate. Figure 4.2 shows the effect of increasing cellulase enzyme loading from 0 ml up to 1.5 ml. there was an increment in glucose production when the enzyme loading was increased from 0 ml until 1.0 ml. Above 1.0 ml, no further increment of glucose production can be seen when the enzyme loading was increased. This is a standard saturation curve for an enzymatic hydrolysis with an excess of substrate. When substrate is in excess as compare to the enzyme molecule, increasing of enzyme molecule will increase the substrate conversion or product producing rate. When the enzyme molecules added has come to the extent that exceeding amount of substrates, further addition of enzyme molecule will not increase the substrate conversion rate. Thus, become the limitation of the enzymatic hydrolysis. As a consequent, a saturation curve as that in Figure 4.2 would be obtained. In conclusion, enzyme loading of 1.0 ml was required to fully hydrolyzed 10 g of newspaper into 0.43 g/L of glucose.

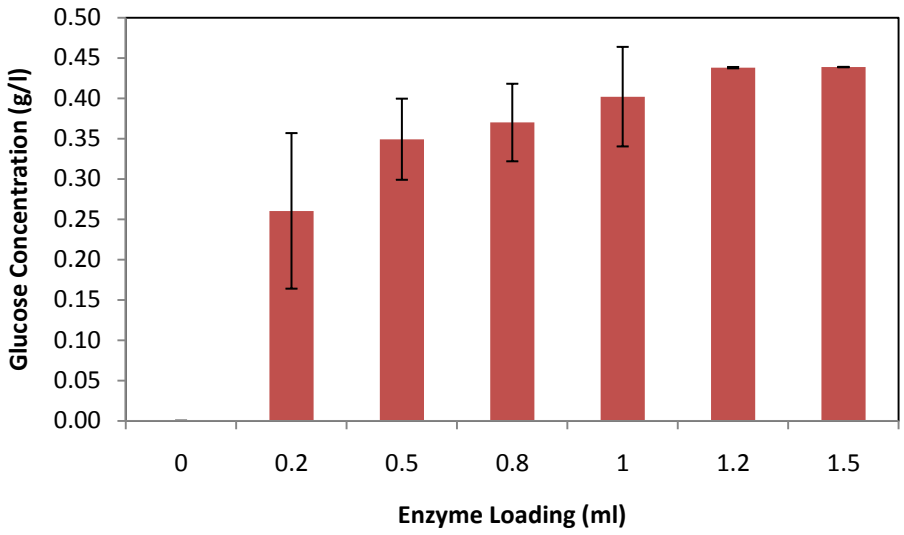

Figure 4.2 Effect of enzyme loading on glucose production

4.4 pH Buffer

The pH for optimum enzyme reaction is typically fallen under neutral region of pH 7. However, there are also enzymes that performed optimally at pH that is more acidic or more basic. To investigate the optimum reaction pH for Novozyme Cellulase, this was performed at a citrate buffer pH range of 3 to 8 using the optimum surfactant ratio obtained in section 4.2 and optimum enzyme loading obtained in section 4.3.

Figure 4.3 illustrate that citrate buffer at pH 6 was the optimum pH for enzymatic hydrolysis of newspaper based on the higher glucose production compared to other pH values. From this study, it shows that the cellulase enzyme is an acidic enzyme because

the glucose production at pH 5 was more than glucose production during pH 7 and pH 8. The cellulase enzyme does not work well at alkaline and at extreme acidic pH.

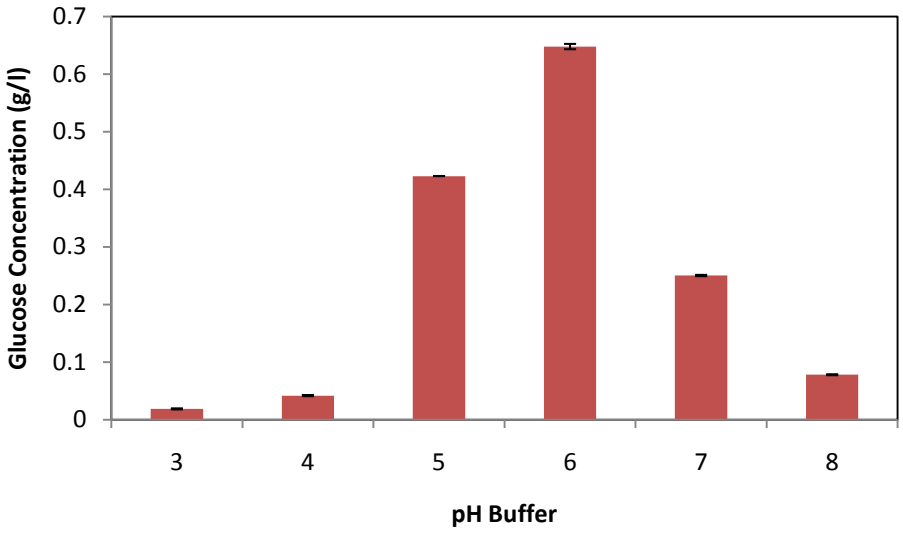

Figure 4.3 Effect of buffer's pH on the glucose

According to Chu and Feng (2012), newspaper pretreated by phosphoric acid resulted in citrate buffer containing cellulase enzyme at pH 4.8 give improvement in glucose production. Furthermore, it also strongly supported by Kim *et. al.* (2007), where enzymatic digestibility of the pretreated substrate in the buffer solution at pH 4.8 would give high glucose production. Besides that, van Wyk and Mohulatsi (2002) stated that the cellulase enzyme performed best in sodium citrate buffer at pH 4.5. All the authors reported an optimum enzymatic hydrolysis pH of acidic region, ranging from pH 4.5 to 4.8. The results obtained in this study revealed that optimum reaction pH for Novozyme

cellulase was at pH 6, which was contradicted with the previous finding. However, it has proved that the cellulase is an acidic enzyme.

4.5 Incubation Temperature

Temperature is an important factor that influencing enzymatic hydrolysis because it determines whether the enzyme reaction is slow or fast. In this study, all the optimum parameters from the previous study (section 4.2 – 4.4) were applied, the incubation temperature was varied between 25 ^{o}C to 80 ^{o}C. Figure 4.4 shows that the highest amount of glucose was produced at 50^{o}C, which was 0.673 g/L. The enzyme reaction becomes slower when the temperature increased above 50 ^{o}C. According to Mussatto *et. al.* (2008) and Selig *et. al*. (2008), the suitable reaction temperature for cellulase enzyme was at 50 ^{o}C. Therefore, the result obtained was in accordance to the finding of others. Optimum temperature for enzymatic reaction normally fall within 40 – 50 ^{o}C, above this range enzyme denaturation occurs. Below this range, the reaction is very much lower.

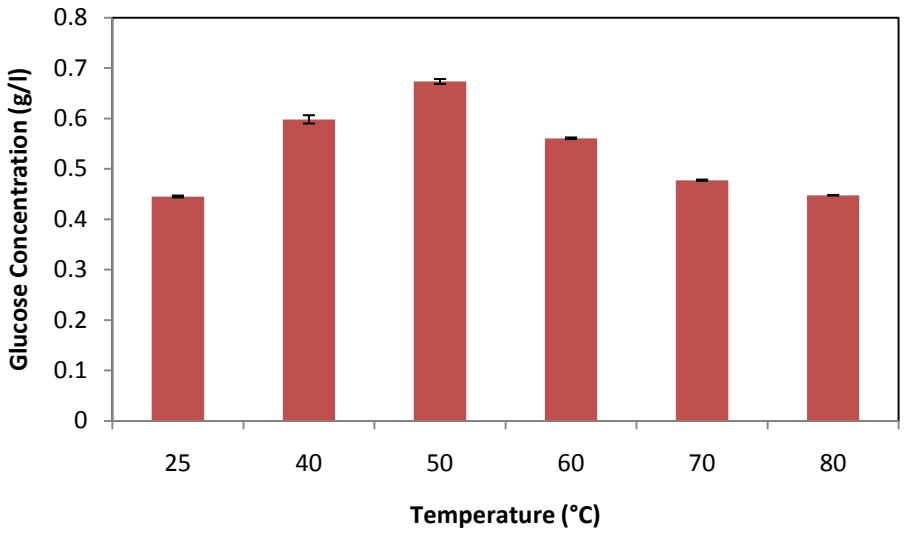

Figure 4.4 Effect of incubation temperature on the glucose production

This study did not used temperature 90 ^{o}C and above because the enzyme will be inactivated and no reaction could be occurred. As had been stated by Andreaus *et. al.* (1999), the catalytic activity of the cellulase was almost completely inhibited at higher pretreatment temperature, which is above 50 ^{o}C.

4.6 Agitation Speed

One of the physical factors that would enhance the enzymatic reaction is agitation. Previous study applied agitation speed as high as 200 rpm for enzymatic

hydrolysis. During this study, the effect of optimum agitation that would give the highest glucose production was identified by varying the speeds from 0 to 400 rpm. Figure 4.6 show that the agitation speed at 200 rpm gave the highest glucose production at a value of 0.62 g/L. According to Azevedo *et. al.* (2000), the mechanical agitation gave effect to the enzyme reaction because it causes substrate become more fibrillation and more exposed to specific surface area for enzyme attack. They found that by using cellulolytic enzyme to condition the fiber will have a greater effect on the fiber after mechanical refining. When the speed of agitation was increased above 200 rpm, the glucose production decreased to 0.55 g/L at 400 rpm. This is because at higher speed, some of the enzyme may denatured and unable to react with the substrate.

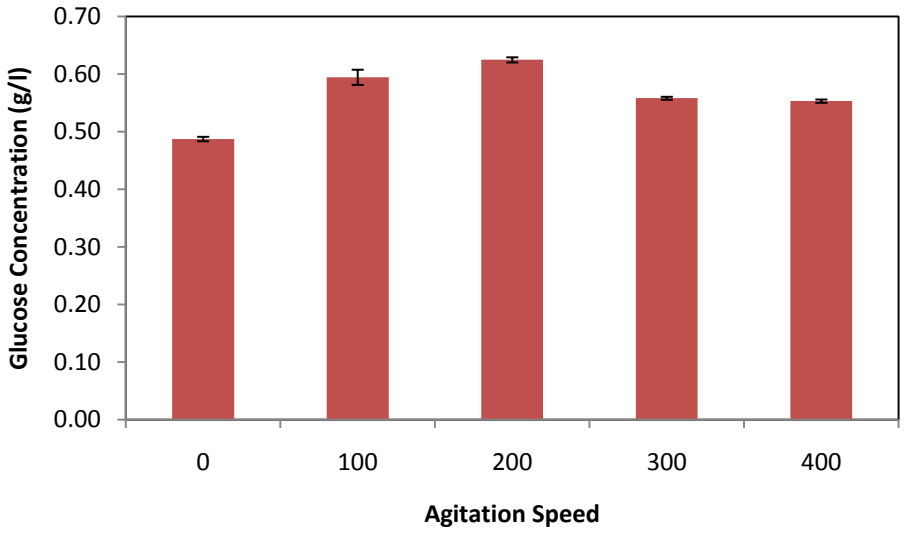

Table 4.5 Effect of agitation speed on the glucose production

4.7 Incubation Time

The glucose production is supposed to increase if longer incubation time was used during enzymatic hydrolysis. According to MacLellan (2010), the hydrolysis of newspaper only required 24 hours at temperatures below 50 ^{o}C using concentrated-acid hydrolysis process. In contrasts, Sun and Cheng (2002) reported that cellulose enzyme required 48 to 72 h to convert cellulose in newspaper into high concentration of glucose. Enzymes produced by a variety of microorganism prefer longer retention time for reaction (Kumar *et. al.*, 2009). It can be seen that different researcher reported different length of incubation time for enzyme hydrolysis. As such, the optimum incubation time for the cellulase used in this study must be investigated.

This study has determined the effect of incubation time in glucose production from 24 h until 120 h. The trend in Figure 4.7 shows that the glucose production was increased when the times of incubation were increased, but during time 96 h until 120 h the line seems to flatten. Furthermore the standard deviation obtained in table 4.7 for incubation time 96 h was 0.73 ± 0.02 g/L, which was equivalent with the incubation time of 120 h (0.75 ± 0.00 g/L). Therefore, further increased in incubation time would not increase glucose production. When all the substrate has been converted to glucose, further increased in incubation time or abundant availability of enzyme would not increase further the glucose production. As a result, incubation time of 96 h seems to be the optimum incubation time for the amount of substrate used.

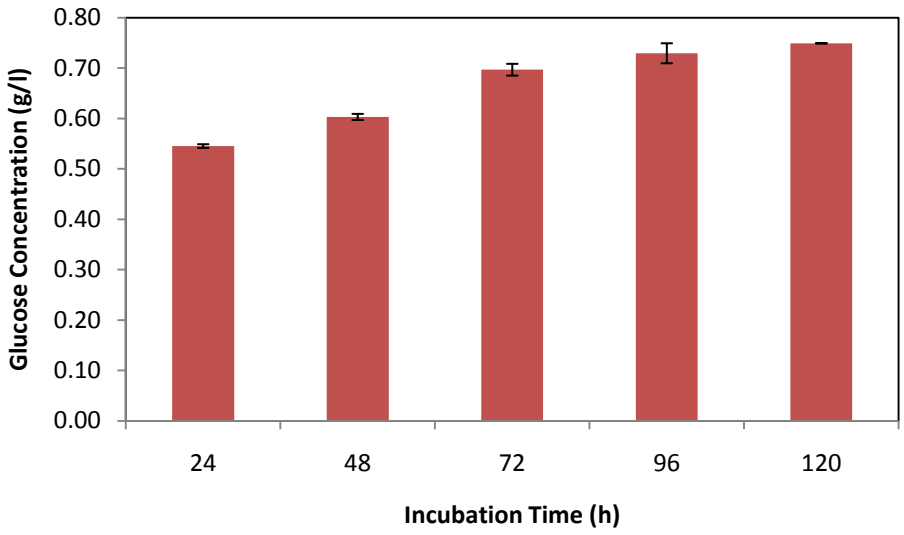

Table 4.6 Effect of incubation time on the glucose production

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

In conclusion, the effect of various operating parameters during enzymatic hydrolysis of newspaper to fermentable glucose by cellulase was been achieved. All parameters were studied where surfactant ratio at 0.2:0.8 of TX-21: TWEEN-80 would give the highest glucose production than other ratios. 1.0 mL cellulase enzyme in citrate buffer at pH 6.0 incubated at 50 ^{o}C and agitated at 200 rpm for 96 hours would produce the highest amount of glucose. The highest glucose obtained in this study was 0.73 ± 0.02 g/L.

5.2 Recommendations

It is recommended to optimize the enzymatic hydrolysis of old newspaper by Novozyme cellulase using RSM because it using mathematical and statistics for empirical model building that design of experiment to optimize the response since the optimum range of the important parameters had been determined in this study.

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APPENDIX A – Citrate Buffer

A.1 Preparation Citrate Buffer

1. 0.9g of citric acid diluted in 50 mL of deionized water
2. 1.8 g of Trisodium citrate anhydrous diluted in 50 mL of deionized water
3. Using dropper, the buffer pH adjusted by mixed the solution of citric acid and trisodium citrate

APPENDIX B – Glucose Determination

B.1 DNS Solution

Weighed the 10 g of 3,5-dinitrosalicyclic acid, 2 g of phenol, 0.5 g of sodium sulphate, 10 g of sodium hydroxide. Those chemicals were mixed by stirred and heated in 500 mL of distilled water until completely dissolved. The solution was cooled down and added

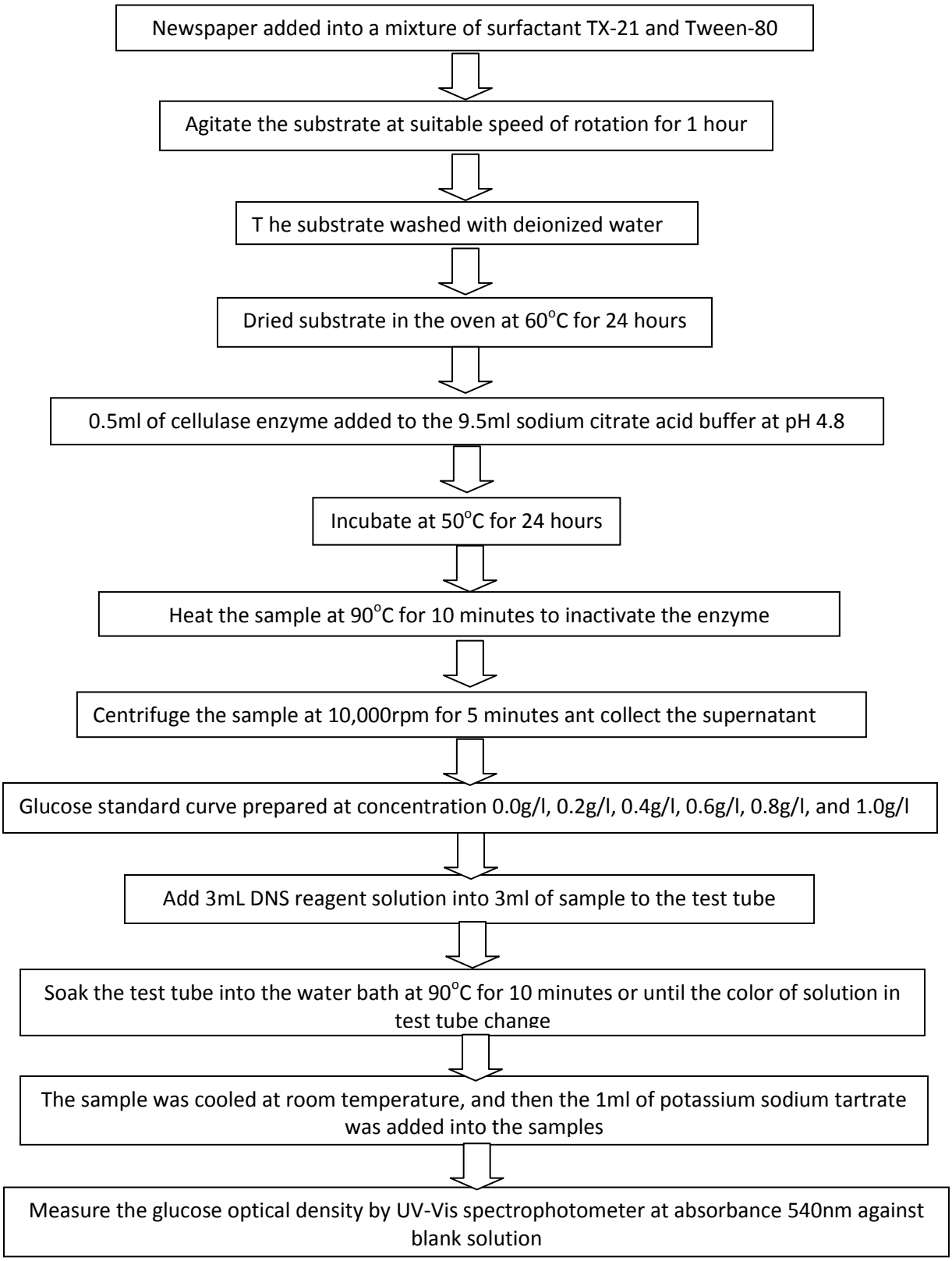
the water up to 1 L. The bottle covered with aluminium foil to prevent light and ensure the cap was closed tightly to avoid entering of carbon dioxide.

APPENDIX C – DNS Method

C.1 DNS Method

1. Prepare glucose solution at the concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L.
2. Add 3 ml of DNS reagent to 3 ml of the glucose solution above in the test tubes. Mix properly.
3. Place all tubes in a boiling water bath for exactly 10 min.
4. Cool down to room temperature.
5. Pipette 1 ml of potassium sodium tartrate into each tube and mix properly.
6. Measure the absorbance at 540 nm using UV-Vis spectrophotometer.

APPENDIX D – General Procedure



APPENDICES E - Results

E.1 Surfactant Ratio

Table E.1.1 Optical density of glucose production from surfactant ratio at 540nm.

Surfactant Ratio	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard Deviation
0.2:0.8	1.28	1.26	1.27	1.27	0.01
0.4:0.6	1.02	1.03	1.03	1.03	0.00
0.5:0.5	0.89	0.80	0.92	0.87	0.06
0.6:0.4	1.14	0.87	1.22	1.07	0.18
0.8:0.2	1.22	0.93	1.02	1.06	0.14
1.0:0	1.21	1.13	1.13	1.15	0.05
0.0:1.0	1.06	1.06	0.98	1.03	0.04
No surfactant	0.64	0.62	0.67	0.64	0.03

Table E.1.2 Glucose standard curve for surfactant ratio

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.34
0.4	0.80
0.6	1.62
0.8	2.08
1	2.21

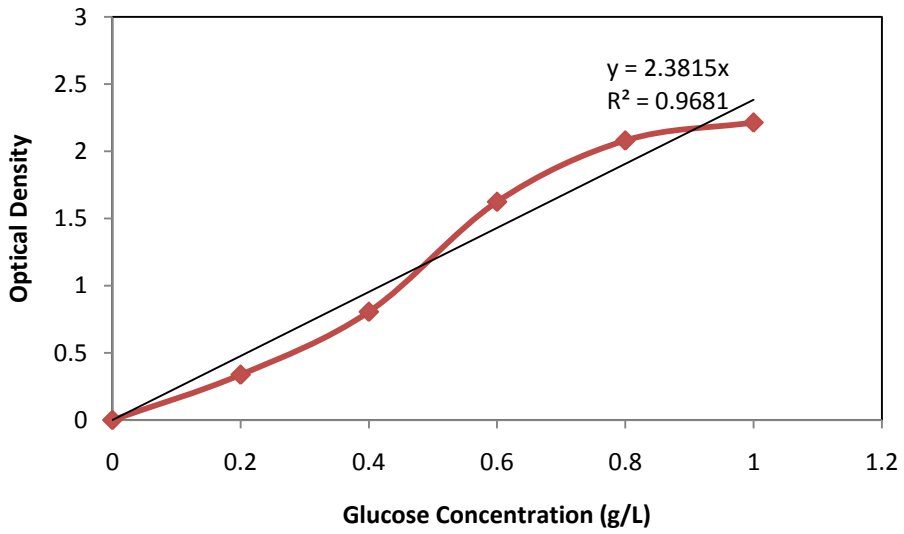


Figure E.1 Glucose standard curve that be used for determine glucose concentration from samples of surfactant ratios

Table E.1.3 Glucose concentration of samples surfactant ratio obtained from glucose standard curve.

Surfactant Ratio	Glucose concentration (g/L) 1	Glucose concentration (g/L) 2	Glucose concentration (g/L) 3	Glucose Concentration (g/L) Average	Glucose Concentration (g/L) Standard Deviation
0.0:1.0	0.45	0.44	0.41	0.43	0.02
0.2:0.8	0.54	0.53	0.53	0.53	0.00
0.4:0.6	0.43	0.44	0.43	0.43	0.00
0.5:0.5	0.37	0.34	0.39	0.37	0.03
0.6:0.4	0.48	0.36	0.51	0.45	0.08
0.8:0.2	0.51	0.39	0.43	0.44	0.06
1.0:0	0.51	0.47	0.47	0.49	0.02
no surfactant	0.27	0.26	0.28	0.27	0.01

E. 2 Enzyme Loading

Table E.2.1 Optical density of glucose production from enzyme loading at 540nm

Enzyme Loading (mL)	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard Deviation
0	0	0	0	0.00	0.00
0.2	1.25	0.56	1.08	0.97	0.36
0.5	1.43	1.08	1.37	1.30	0.19
0.8	1.45	1.17	1.50	1.37	0.18
1.0	1.59	1.23	1.65	1.49	0.23
1.2	1.63	1.62	1.63	1.63	0.00
1.5	1.63	1.63	1.63	1.63	0.00

Table E.2.2 Glucose standard curve for enzyme loading

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.79
0.4	1.55
0.6	2.16
0.8	2.45
1	2.58

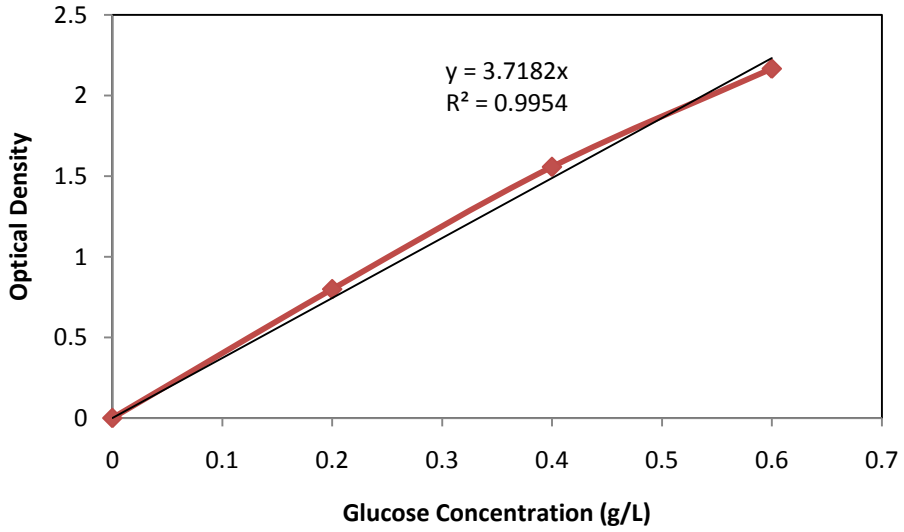


Figure E.2 Glucose standard curve that be used for determine glucose concentration from samples of enzyme loading

Table E.2.3 Glucose concentration of samples enzyme loading obtained from glucose standard curve

Enzyme Loading (mL)	Glucose Concentration (g/L) 1	Glucose Concentration (g/L) 2	Glucose Concentration (g/L) 3	Glucose Concentration (g/L) Average	Glucose Concentration (g/L) Standard Deviation
0	0.00	0.00	0.00	0.00	0.00
0.2	0.34	0.15	0.29	0.26	0.10
0.5	0.39	0.29	0.37	0.35	0.05
0.8	0.39	0.31	0.40	0.37	0.05
1	0.43	0.33	0.45	0.40	0.06
1.2	0.44	0.44	0.44	0.44	0.00
1.5	0.44	0.44	0.44	0.44	0.00

E.3 pH buffer

Table E.3.1 Optical density of glucose production from pH buffer at 540nm

Buffer's pH	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard deviation
3	0.05	0.05	0.06	0.05	0.00
4	0.13	0.12	0.12	0.13	0.00
5	1.28	1.28	1.28	1.28	0.00
6	1.96	1.94	1.97	1.96	0.01
7	0.76	0.76	0.75	0.75	0.00
8	0.24	0.2	0.23	0.23	0.00

Table E.3.2 Glucose standard curve for pH buffer

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.81
0.4	1.49
0.6	2.13
0.8	2.45
1	2.65

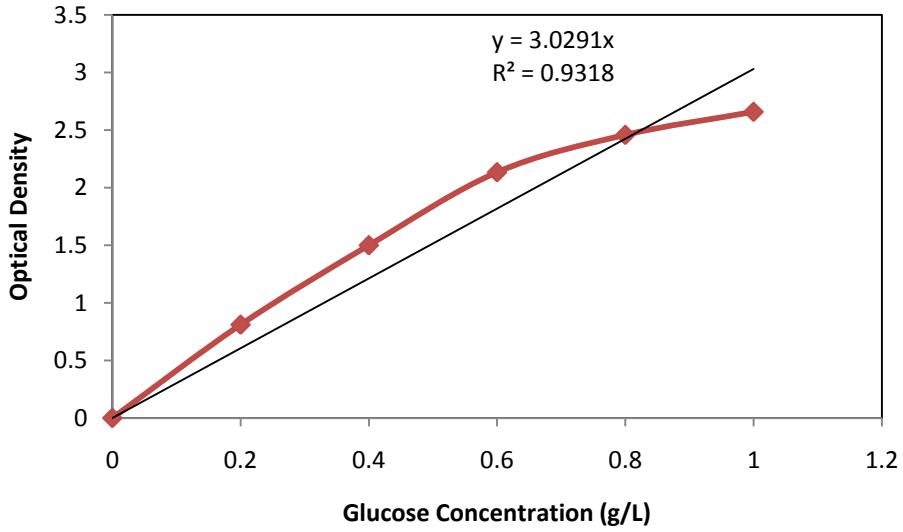


Figure E.3 Glucose standard curve that be used for determine glucose concentration from samples of enzyme loading

Table E.3.3 Glucose concentration of samples enzyme loading obtained from glucose standard curve

Buffer's pH	Glucose Concentration (g/L) 1	Glucose Concentration (g/L) 2	Glucose Concentration (g/L) 3	Glucose Concentration (g/L) Average	Glucose Concentration (g/L) Standard Deviation
3	0.01	0.02	0.02	0.02	0.00
4	0.04	0.04	0.04	0.04	0.00
5	0.42	0.42	0.43	0.42	0.00
6	0.64	0.64	0.65	0.64	0.00
7	0.25	0.25	0.25	0.25	0.00
8	0.07	0.08	0.08	0.07	0.00

E.4 Incubation Temperature

Table E.4.1 Optical density of glucose production from incubation temperature at 540nm.

Incubation Temperature (^{o}C)	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard Deviation
25	1.30	1.29	1.29	1.29	0.01
40	1.75	1.71	1.76	1.74	0.02
50	1.96	1.94	1.97	1.96	0.01
60	1.63	1.62	1.63	1.63	0.00
70	1.38	1.39	1.39	1.39	0.00
80	1.30	1.30	1.30	1.30	0.00

Table E.4.2 Glucose standard curve for incubation temperature

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.88
0.4	1.39
0.6	1.96
0.8	2.32
1	2.63

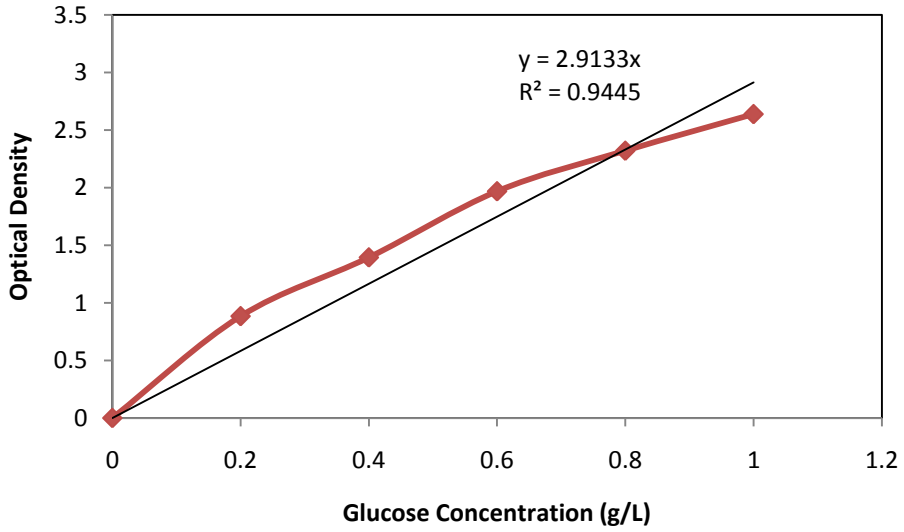


Figure E.4: Glucose standard curve that be used for determine glucose concentration from samples of incubation temperature

Table E.4.3 Glucose concentration of samples incubation temperature obtained from glucose standard curve

Incubation Temperature (^{o}C)	**Glucose Concentration (g/L) 1**	**Glucose Concentration (g/L) 2**	**Glucose Concentration (g/L) 3**	**Glucose Concentration (g/L) Average**	**Glucose Concentration (g/L) Standard deviation**
25	0.44	0.44	0.44	0.44	0.00
40	0.60	0.58	0.60	0.59	0.00
50	0.67	0.66	0.67	0.67	0.00
60	0.56	0.55	0.56	0.56	0.00
70	0.47	0.47	0.47	0.47	0.00
80	0.44	0.44	0.44	0.44	0.00

E.5 Agitation Speed

Table E.5.1 Optical density of glucose production from agitation speed at 540nm.

Speed (rpm)	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard Deviation
0	1.52	1.52	1.54	1.53	0.01
100	1.81	1.88	1.89	1.86	0.04
200	1.96	1.94	1.97	1.96	0.01
300	1.75	1.75	1.74	1.75	0.00
400	1.73	1.74	1.72	1.73	0.00

Table E.5.2 Glucose standard curve for agitation speed

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.79
0.4	1.45
0.6	1.92
0.8	2.34
1	2.56

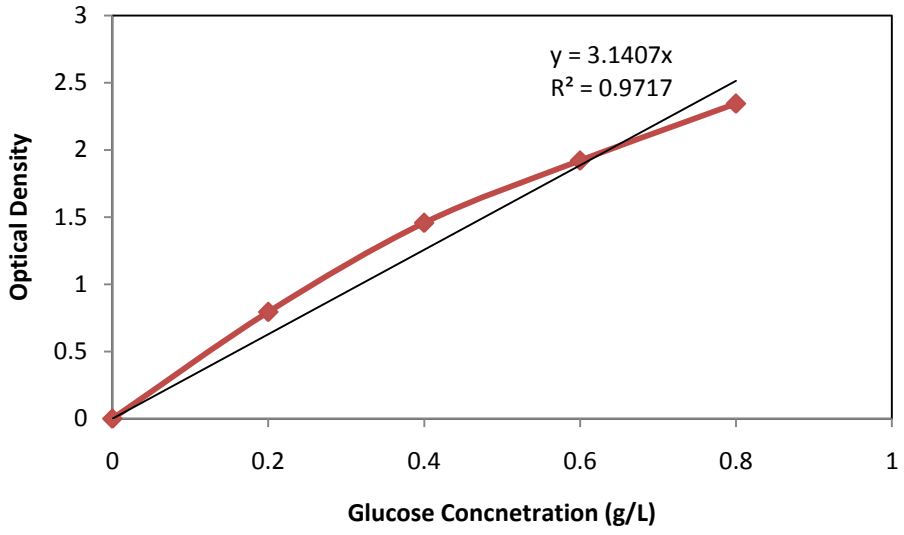


Figure E.5: Glucose standard curve that be used for determine glucose concentration from samples of agitation speed

Table E.5.3 Glucose concentration of samples agitation speed obtained from glucose standard curve

SPEED (rpm)	Glucose Concentration (g/L) 1	Glucose Concentration (g/L) 2	Glucose Concentration (g/L) 3	Glucose Concentration (g/L) average	Glucose Concentration (g/L) standard deviation
0	0.48	0.49	0.49	0.49	0.00
100	0.58	0.60	0.60	0.59	0.01
200	0.63	0.62	0.63	0.62	0.00
300	0.56	0.56	0.55	0.56	0.00
400	0.55	0.56	0.55	0.55	0.00

E.6 Incubation Time

Table E.6.1 Optical density of glucose production from incubation time at 540nm

Incubation Time (h)	Optical Density 1	Optical Density 2	Optical Density 3	Optical Density Average	Optical Density Standard Deviation
24	1.78	1.8	1.81	1.79	0.01
48	1.97	1.99	2.01	1.99	0.02
72	2.26	2.30	2.33	2.29	0.03
96	2.46	2.42	2.33	2.40	0.06
120	2.47	2.47	2.47	2.47	0.00

Table E.6.2 Glucose standard curve for incubation time.

Glucose Concentration (g/L)	Optical Density
0	0
0.2	0.81
0.4	1.49
0.6	2.04
0.8	2.46
1	2.68

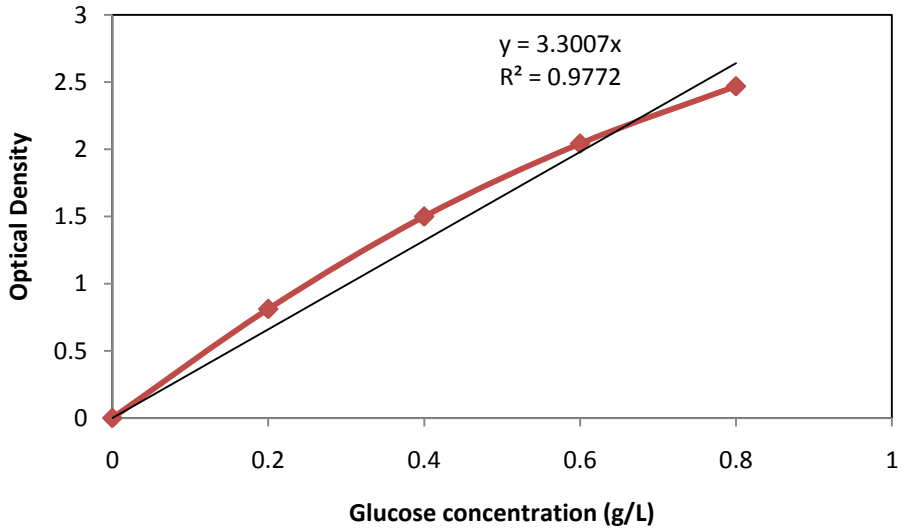


Figure E.6 Glucose standard curve that be used for determine glucose concentration from samples of incubation time.

Table E.6.3 Glucose concentration of samples incubation time obtained from glucose standard curve.

Incubation Time (h)	Glucose concentration (g/L) 1	Glucose concentration (g/L) 2	Glucose concentration (g/L) 3	Glucose Concentration (g/L) Average	Glucose concentration Standard Deviation
24	0.54	0.55	0.55	0.55	0.00
48	0.60	0.60	0.61	0.60	0.01
72	0.68	0.70	0.71	0.70	0.01
96	0.75	0.73	0.71	0.73	0.02
120	0.75	0.75	0.75	0.75	0.00