STUDY ON THE EFFECT OF ULTRASOUND ON CELLULOSE HYDROLYSIS BY CELLULASE

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ABSTRACT

Cellulose is a natural polymer that has a potential of utilization of cellulosic biomass as a renewable resource for reducing emissions of carbon dioxide and to be used as future fuels such as ethanol and other chemical products. The study on the effect of ultrasound on cellulose hydrolysis by cellulase was to be conducted. The purpose of the study is to determine the optimum condition of sonication regimen in enzymatic hydrolysis of cellulose, determine the best parameters of sonication powers and duty cycle for enzymatic hydrolysis using Michaelis-Menten kinetics and study the effect of substrate particle size (sigmacell cellulose) on the rate of reaction (solid liquid mass transfer effect). The method of this research include the preparation of substrate by dissolving the powder in 500ml of 0.05 M acetate buffer, pH 4.8, hydrolysis of cellulose in a 2 L stirred beaker, sonication amplitude for ultrasound-assisted hydrolysis, testing the cellulase stability and activity, and also Dinitrosalicylic Acid (DNS) method for analysis. From the experiment, it can be conclude that both hydrolysis of soluble and insoluble cellulose followed Michealis-Menten kinetics model. Besides that, it is proved that sonication always enhances rate of product formation regardless of substrate particle size. In contrast, an increasing particle size reduced the rate of hydrolysis regardless of implied sonication.

KAJIAN MENGENAI KESAN ULTRSOUND TERHADAP HIDROLISIS CELLULOSE MENGGUNAKAN ENZIM CELLULASE

ABSTRAK

Selulosa adalah polimer semulajadi dan sebagai biojisim, ia mempunyai potensi sebagai sumber yang boleh diperbaharui untuk mengurangkan pelepasan karbon dioksida dan boleh digunakan sebagai bahan api seperti etanol dan bahan kimia lain di masa hadapan. Kajian mengenai kesan ultrasound terhadap hidrolisis selulosa oleh selulase telah dijalankan. Antara tujuan utama kajian ini dijalankan adalah untuk menentukan keadaan optimum untuk regimen ultrasound semasa hidrolisis enzim keatas selulosa, menentukan parameter seperti kuasa ultrasound dan kitaran yang terbaik untuk hidrolisis enzim dengan menggunakan model kinetic Michaelis-Menten dan untuk mengkaji kesan perbezaan saiz zarah substrat terhadap kadar tindak balas (kesan pemindahan jisim antara pepejal dan cecair). Antara kaedah yang digunakan sewaktu menjalankan kajian ini termasuk penyediaan substrat dengan melarutkannya di dalam larutan buffer asetik yang mempunyai pH 4.8 dan kepekatan 0.05 M, hidrolisis selulosa di dalam balang 2 L yang dikacau, mengaplikasikan ultrasound pada kuasa intensity dan kitaran tertentu, menguji kestabilan dan aktiviti selulase, dan juga menganalisa produk menggunakan kaedah asid dinotrosalicylic (DNS). Daripada eksperimen yang telah dijalankan, didapati kedua-dua hidrolisis selulosa larut dan tidak larut menepati model kinetic Michaelis-Menten. Selain itu, ultrasound telah terbukti dapat meningkatkan kadar pembentukan produk tanpa mengira saiz zarah substrat. Sebaliknye, peningkatan saiz zarah terbukti telah mengurangkan kadar hidrolisis tanpa mengira kuasa ultrasound yang diaplikasikan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABREVIATIONS

°C	Degree Celsius
g	Gram
K_M	Michaelis Constant
mM	Milimol
L	Liter
Rpm	Rotation per Minute
S_o	Initial Substrate concentration
V_o	Initial rate of reaction
V_{max}	Maximum rate of reaction
%	Percentage
Wcm^{-2}	Waltz per centimeter square

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cellulose is the most abundant natural polymer on Earth. It has a great potential to be converted into its monomeric units, glucose, which can then be used to produce future fuels such as ethanol, other chemical products, and even electricity through fuel cells (Saqib and Whitney,2006). In other research carried out by Li *et al* (2007) they stated that the study of enzymatic hydrolysis of cellulose was very extensive in past decades since the potential of utilization of lignocellulosic biomass as a renewable resource for reducing emissions of carbon dioxide and thereby prevents global warming was known.

During the past years, many reserch has been done on converting lignocellulosic biomass into bioethanol with the aim to produce the 2nd generation biofuel. The most common processing of lignocellulosics to bioethanol consists of four major unit operations: pretreatment of raw materials, enzymatic hydrolysis of pretreated materials into fermentable sugars, fermentation of fermentable sugars into ethanol, and ethanol separation or purification (Lin *et al*,2010). Among the four major unit operations, enzymatic hyrolysis of lignocellulosic biomass process play a very important role that effect the cost of bioethanol production. During 1970s, eventhough the reserch on biological conversion of lignocellulosic biomass to fuels and chemical has the potential of low cost and higher yield and selectivity, this technologies were believed to be too high risk for industry to applied at that time.

However, due to its complex structure, enzymatic hydrolysis of cellulose almost become impossible. This has been state in the research done by Van Wyk, 1997 which he highlight that heterogeneous enzymatic hyrolysis of cellulose by cellulase is a complex process and adsorption tendencies of cellulases on cellulose could be seen as the most difficult part of the reaction. Agree with Van Wyk, Yang *et al*,2011 clearly state that enzymatic hydrolysis that converts lignocellulosic biomass to fermentable sugars may be the most complex step in this process due to substrate-related and enzyme-related effects and their interactions.

Therefore, to achieve an effective hydrolysis of lignocellulosics, it is necessary and important to deeply understand whether enzyme are weak lignin-binding or strong lignin-binding, and whether the different combinations of biomass components in substrates have a significant influence on enzyme activity during hydrolysis of biomass wastes.

The one that responsible for the bioconversion of cellulose into soluble sugar during enzymatic hydrolysis is cellulase. In nature, many cellulases are involved in cellulose transformation. Cellulase is a multicomponent enzyme consist of three different enzymes which are, endocellulases, cellobiohydrolase, and β-glucosidases (Li et al, 2007) as shown in Figure 2. It is a macromolecule that depending on the number of monomer units in its structure, may be fully soluble in water, or may occur as insoluble particles.Besides that, according to Li *et al* (2007), cellulases usually were applied in fabric modification, paper and pulp industry, and food industry.

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives, and ash (Lin *et al*,2010). The example of biomass feedstock include agriculture residue, woody crops, and municipal solid waste (Yang *et al*,2011).

1.2 Problem Statement

Nowadays, researcher paid a special attention on the ability of biomass to achieve global sustainability (Collinsona and Thielemans,2010). Cellulose is a very important renewable biomass resource since sooner or later human being will be facing the shortage of fossil fuels problems. In views of its low cost, abundance and renewability, cellulose is an important resource that can potentially provide huge

quantities of glucose to manufacture of bioethanol and many other chemicals by fermentation.

Enzyme mediated hydrolysis of cellulose with and without ultrasound, offers an opportunity to investigate the effect of substrate molecule size and solid-liquid mass transfer effects in a bioreactor.

The use of ultrasound in enzymatic hydrolysis of cellulose has been explored by many researchers (Gama *et al,*1997; Galesio *et al,*2012; Kwiatkowska,2011; Luque *et al,*2011). According them, prolonged or intense ultrasound can damage cellulase, therefore selection of suitable sonication regimen is important in an enzymatic hydrolysis process.

Use of ultrasound is considered to be preferable because of its simpler equipment and mild operating condition for the enzymatic hydrolysis. Thus, in this proposal the effect of low intensity ultrasound on the enzymatic hydrolysis of cellulose will be investigate using both soluble and suspended particulate cellulose.

1.3 Objectives of Study

Based on the background of this study, the objectives of this study are listed as following:

1.3.1 To determine the optimum condition of sonication regimen in enzymatic hydrolysis of cellulose.

1.3.2 To determine the best parameters of sonication powers for enzymatic hydrolysis using Michaelis-Menten kinetics.

1.3.3 To study the effect of substrate particle size on the rate of reaction (solid liquid mass transfer effect).

1.4 Scope of Study

Based on the objectives of this study, the scopes of study are declared as follows:

1.4.1 To determine the yield of glucose by UV-Vis before and after applying ultrasound.

1.4.2 To find the best sonication regiment by applying different duty cycle sonication.

1.4.3 To test the result by comparing the yield of glucose before and after applying ultrasound for different substrate particle size.

1.5 Significance of Study

It is necessary to develop new fundamental strategies to solve a wastes problem that still not been solve till now. Waste reutilization has become a matter of great interest since the increased waste emission is threatening the limited resources and living spaces on the Earth. Since the major component of domestic solid waste such as paper and plastic mainly contain cellulose, this cellulose can be hydrolyze to reducing sugar that used to produce bioethanol. Moreover, bioethanol that is ethanol made microbially from biomass, such as cellulose has been approved as an alternative energy source for increasing energy security and reducing air pollution from contaminants such as nitrogen oxide, NOx (Li *et al*,2005).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Cellulose.

Cellulose is the most abundant carbohydrate produced by plant and also a polymer of glucose. Cellulose can be soluble and insoluble, which according to current understanding, have no clear common properties (Lindman, Karlström and Stigsson, 2010) and is not melt processible because it decompose before it undergoes melt flow. Cellulose is a linear polymer of β-glucoses linked by 1,4-glucosidic bonds (Xu,Ding and Tejirian,2009). According to Xu *et al*,2009, the free hemi-acetal (or aldehyde) at the C-1 site of the "reducing" end is quite reactive towards oxidation, the hydroxyls at the C-6 sites of the anhydroglucosyl units are less reactive, and the rest of hydroxyls, including that at the C-4 site of the "nonreducing" end, are the

least reactive, thus make cellulose oxidation-prone, and their oxidation during biomass pre-treatments may alter cellulose's property. The structure of cellulose were as shown in Figure 2.1.

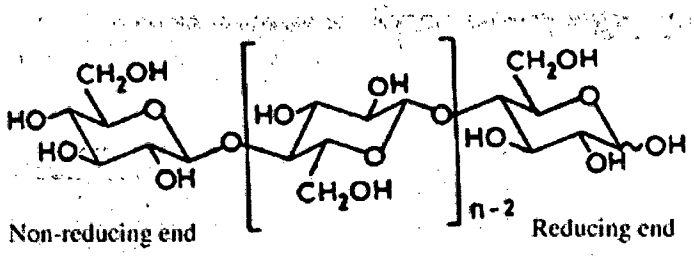

Figure 2.1 Cellulose structure. [**Source**: http://www.fibersource.com].

2.2 Enzyme Substrate

According to Paljevac *et al* (2007), cellulases are the enzymes which hydrolyse the β-1,4-linkages in cellulose and are found in many of the sequence-based families of glycoside hydrolases. Cellulase composed of endo-1,4-β-D-glucanases or endoglucanases, exo-1,4-β-D-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases, which work in a interactive manner for hydrolysis of cellulose. Endoglucanases initiate cellulose hydrolysis process, disrupting internal β-1,4-glucosidic bonds along the cellulose chain, increasing the number of ends of cellulose chains available for exoglucanases, take place predominantly in the amorphous regions of cellulose, then the exoglucanases cleave off two units (cellobiose) from each end of these shorter cellulose chains before glucosidases

hydrolyze the disaccharides cellobiose units into two monosaccharide (glucose) units (Ogeda *et al*, 2012).

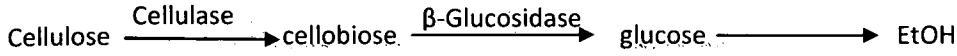

Figure 2.2 Sequence from cellulose to biofuels [**Source:** Bommarius *et al*,2008]

2.3 Type of Reaction Involved

There will be two classes of reaction involve in this research, that is homogenous and heterogenous reaction.

2.3.1 Homogenous reaction

Homogenous reaction is any of a class of chemical reactions that occur in a single phase (gaseous, liquid, or solid), based on the physical state of the substances present. According to the theory-based, homogeneous reactions are the simpler of the two classes of reactions because the chemical changes that take place are solely dependent on the nature of the interactions of the reacting substances

(www.britannica.com). In this research, the reaction that involve homogenous reaction is the reaction between soluble cellulose substrate with soluble enzyme.

2.3.2 Heterogenous reaction

While for heterogenous, it is define that any of a class of chemical reactions in which the reactants are components of two or more phases (solid and gas, solid and liquid, two immiscible liquids) or in which one or more reactants undergo chemical change at an interface. The reaction of metals with acids, the electrochemical changes that occur in batteries and electrolytic cells, and the phenomena of corrosion are part of the subject of heterogeneous reactions. The majority of the researches on heterogeneous reactions are devoted to heterogeneous catalysis for example the reactions between gases or liquids accelerated by solids (www.britannica.com). The reaction between soluble carboxymethyl cellulose with cellulase is the example of heterogenous reaction in this research.

2.4 Enzymatic Hydrolysis.

The common methods for degradation of cellulose to glucose are acid hydrolysis and enzymatic hydrolysis. Yang *et al*, 2011 define enzymatic hydrolysis as multi-step heterogeneous reaction in which insoluble cellulose is initially broken down at the solid–liquid interface via the synergistic action of endoglucanases and exoglucanases/cellobiohydrolases. This initial reaction is accompanied by further liquid-phase hydrolysis of soluble intermediates, that is, short cellulooligosaccharides and cellobiose, which are catalytically cleaved to produce glucose by the action of β-glucosidase.

The hydrolysis of cellulose by mineral acids is strongly affected by the acid concentration and temperature and mineral acid hydrolysis yields byproducts that are fermentation inhibitors (Shaikh *et al*,2011).

Shaikh *et al* (2011) in their research quoted that if enzymes are to be used for hydrolysis of cellulose, various factors play important roles such as physical properties of the substrate, composition of substrate, crystallinity of cellulose, degree of polymerization, enzyme complex synergy, bulk and pore diffusion, and kinetics. In past research conduct by Zhong *et al* (2007) cited the most prefereble methods was enzymatic hydrolysis because instead of can avoid using toxic and corrosive chemicals, at the same time it can economize energy on account of the relatively mild reaction conditions. Besides that, enzymatic hydrolysis that was carried out at room temperature will gives colourless pure product and reduces the byproduct formation due to enzyme specificity (Rathod, and Pandit,2010).

Shaikh *et al* (2011) also explain the hydrolysis process in detail. According to them, during the hydrolysis of cellulose, the endoglucanases attack the cellulose polymer chain in a random manner creating new reducing ends. This reaction is followed by the hydrolysis with exoglucanases, which attack the cellulose from either end, forming cellobiose. Finally, the β-glucosidase completes the hydrolytic process through the formation of glucose from cellobiose. It can be conclude that all three enzymes work in a interactive manner for hydrolysis cellulose.

Unfortunately, eventhough enzymatic hydrolysis is the best alternative,it also lack in some aspect such as slow reaction rate and high cost of enzyme as cited by Rathod, and Pandit (2010) . Thus, in the later research by Ogeda *et al* (2012) said that the alternative of the high cost enzyme that is by immobilizing cellulase onto solid supports because it can make the enzymatic hydrolysis more competetive because the enzyme can be recycled. Moreover, the success of enzymatic hydrolysis depends on the close contact between cellulase and cellulose.

Enzymatic hydrolysis of cellulose into glucose, which could be fermented into ethanol, isopropanol or butanol, is not yet economically feasible. However, as quote by Pierre and Aubert (1994), the need to reduce emissions of greenhouse gases provides the incentive for the development of processes generating fuels from cellulose, a major renewable carbon source.

2.5 Effect of Ultrasound.

Ultrasound (US) has become a latest technological process in a large variety of scientific fields. The main reason US was develop was beacause it can enhance biological processes or processing such as enzymatic transformations, environmental remediation, fermentations, anaerobic digestion, food processing and enzyme assisted chemical synthesis. According to Kwiatkowska *et al* (2011), the sound frequency above 18 kHz is considered to be ultrasound (US) and a huge amount of research has gone into the application of ultrasound at both high and low power. The three US equipment that usually been used were an ultrasonic probe system,an ultrasonic bath, or an ultrasonic transducer fitted to a glass reactor (Kwiatkowska *et al*,2011). Ultrasound irradiation, is an alternative method to reduce mass transfer limitations in enzymatic reactions as ultrasonic actions in liquids can cause effects of cavitation and when cavitation bubbles collapse near the phase boundary of two immiscible liquids, the resultant shock wave can provide a very efficient stirring/mixing of the layers thus can enhance heterogeneous reactions and readily form transient reactive species which make ultrasound very useful tool in enzymatic reactions (Liu *et al*,2008).

Kwiatkowska *et al* (2011) in their research highlight the fact about the application of low-power ultrasound can increases growth in microbial cell cultures but high power will causes cell disruption. Hence, it must be stressed out that the influence of sonic radiation on the activity and stability of enzymes depends on the sonication parameters and the specific enzyme preparation.

Biotechnology was a new advance technology which provided entirely new opportunities for sustainable production of existing and new products and services in various science's field such as medicine, agriculture, material science and chemistry. The use of ultrasound in environmental remedy can be considered as a green technological application particularly when related to bioprocesses.

The application of ultrasound are widely used in physical and chemical process, such as in the area of biology and biochemistry, engineering, dentistry, geography and geology, polymers and plastics. The summary of ultrasound application is listed in Table 2.1.

Table 2.1: Some laboratory and industrial use of ultrasound **[Source:** Yunus, 2012]

Field	Application
Biology, biochemistry	Homogenisation and cell disruption: power ultrasound is used to rupture cell walls in order to release content for further studies.
Engineering	Ultrasound has been used to assisst drilling, grinding, and cutting. It is particularly useful for processing hard, brittle material, e.g. glass, ceramics. Other uses of power ultrasound are welding (both plastics amd metals) and metal tube drawing. High frequency (MHz) ultrasound is uses in non-destructive testing of materials and flaw detection.
Dentistry	For both cleaning and drilling of teeth.
Geography, geology	Pulse/echo techniques are uses in the location of mineral and oil deposits and in depth gauges for seas and oceans. Echo ranging at sea has been used for many years.

Industrial	Pigments and solids can be easily dispersed in paint, inks, and resins. Engineering articles are often cleaned and degreased by immersion in ultrasonic baths. Two less widely used application are acoustic filtration and ultrasound drying.
Medicine	Ultrasound imaging (2-10 MHz) is used , particularly on obstetrics, for observing the foetus and for guiding subcutaneous surgical implements. In physiotheraphy lower frequencies (20-50 kHz) are used in the treatment of muscle strains.
Plastic and polymers	The welding of thermoplastics is effectively achieved using power ultrasound. The initiation of polymerisation and polymer degradation are also affected. Cure rates of resins and their composition can be measured with high-frequency ultrasound.