SYNTHESIS OF XYLITOL FROM *MERANTI* WOOD SAWDUST IN BATCH PROCESS BY LOCALLY PRODUCED XYLOSE REDUCTASE

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Doctor of Philosophy in Bioprocess Engineering

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

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Dedicated to my friendly wife, children and beloved late parents

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ABSTRACT

The microbial production of xylitol has been studied extensively as an alternative to the chemical process, which on an industrial-scale is time-consuming mainly due to sterilization, inoculum development and product recovery problem. Apart from using pure xylose, no other substrate has been proposed for xylitol synthesis using xylose reductase (XR). The aim of this study was to synthesize xylitol, a specialty product, from *Meranti* wood sawdust hemicellulosic hydrolysate (MWSHH) by locally produced XR. MWS is a sawmill waste, which can be a promising source of xylose for xylitol production. The kinetic parameters of mathematical models were determined to predict xylose, glucose, furfural, and acetic acid concentration in the hydrolysate. Optimization of MWS hydrolysis was conducted with statistical design including one-factor-at-a-time (OFAT) method for maximum xylose recovery. The results of OFAT indicated the residence time, temperature, and acid concentration to be the major factors improving xylose recovery with a value of 60 min, 125 ºC, and 4%, respectively. These factors were further optimized by response surface methodology (RSM), and the optimum values obtained were 80 min, 124 ºC, and 3.26%, respectively. Under these conditions xylose yield and selectivity were attained at 90.6% and 4.05 g/g, respectively. XR was isolated from adapted *C. tropicalis* and characterized. The activity of NADPH-dependent XR measured was 11.16 U/mL. It was stable at pH 5.0–7.0 and temperature of 25–40 ºC for 24 h, and retained above 95% of its original activity after 4 months of storage at –80 ºC. The K_m values of XR for xylose and NADPH were 81.78 mM and 7.29 µM while the V_{max} for xylose and NADPH were 178.57 and 12.5 µM/min, respectively. The high V_{max} and low K_m values of XR for xylose reflecting a highly productive reaction among XR and xylose. Further, a sequential optimization based on OFAT approach and statistical program was followed to enhance xylitol production from xylose-rich MWSHH using XR. Firstly, the OFAT method coarsely evaluated the optimum levels of variables. Secondly, the significant variables for maximum xylitol production were screened out through fractional factorial design (FFD) to be reaction time, temperature and pH. Finally, the significant variables were fine-tuned by RSM and were found to be 12.25 h, 35 ºC and 6.5, respectively, giving the maximum xylitol yield and productivity of 86.57% and 1.33 g/L·h, respectively. Optimization of process conditions using sequential strategies resulted in 1.55-fold improvement in overall xylitol synthesis. This study developed a novel reaction medium to improve xylitol production to a considerable level using MWSHH.

ABSTRAK

Pengeluaran xylitol menggunakan mikrob telah dikaji secara meluas sebagai alternatif kepada proses kimia, pada skala industri, memerlukan masa yang panjang terutamanya disebabkan oleh pensterilan, pembangunan inokulum dan masalah pemulihan produk. Selain daripada menggunakan xilosa tulen, tiada substrat lain telah dicadangkan dalam kajian sebelum ini bagi sintesis xylitol menggunakan *xylose reductase* (XR). Tujuan kajian ini adalah untuk menghasilkan xylitol, produk yang istimewa, dari hidrolisat hemiselulosa habuk kayu Meranti (MWSHH) oleh XR. MWS adalah sisa kilang papan, yang boleh menjadi sumber xilosa untuk pengeluaran xylitol. Parameter kinetik model matematik telah ditentukan untuk meramalkan kepekatan xilosa, glukosa, furfural, dan asid asetik dalam hidrolisat. Pengoptimuman hidrolisis MWS telah dijalankan dengan reka bentuk statistik termasuk kaedah satu faktor dalam satu masa (OFAT) untuk pemulihan xilosa maksimum. Keputusan OFAT menunjukkan masa penahanan, suhu, dan kepekatan asid menjadi faktor utama bagi meningkatkan pengekstrakan xilosa dengan nilai masing-masing adalah 60 minit, 125 ºC, dan 4%. Faktor-faktor ini kemudian dioptimumkan dengan kaedah permukaan respons (RSM), dan nilai optimum yang diperolehi masing-masing adalah 80 minit, 124 ºC, dan 3.26%. Di bawah keadaan ini hasil xilosa dan selektiviti telah dicapai masing-masing pada 90.6% dan 4.05 g/g. XR telah dipencilkan daripada *C. tropicalis* dan dibuat pencirian. Aktiviti NADPH bergantung kepada XR yang diukur iaitu 11.16 U/mL. Ia adalah stabil pada pH 5.0–7.0 dan suhu 25–40 ºC selama 24 jam, dan disimpan melebihi 95% daripada aktiviti asal selepas 4 bulan penyimpanan pada –80 ºC. Nilai-nilai K_m XR untuk xilosa dan NADPH adalah 81.78 mM dan 7.29 μM manakala V_{max} untuk xilosa dan NADPH adalah 178.57 dan 12.5 μM/min. Nilai V_{max} XR tinggi dan K_m XR rendah untuk xilosa mencerminkan reaksi yang sangat produktif di antara XR dan xilosa. Seterusnya, pengoptimuman secara berturutan yang berdasarkan pendekatan OFAT dan program statistik dijalankan untuk meningkatkan pengeluaran xylitol dari MWSHH yang kaya dengan xilosa menggunakan XR. Pertama, tahap optimum pembolehubah dinilai secara kasar menggunakan kaedah OFAT. Kedua, pembolehubah penting untuk pengeluaran xylitol yang maksimum telah disaring melalui rekabentuk eksperimen separa faktor (FFD) iaitu masa tindak balas, suhu dan pH. Akhirnya, pembolehubah penting diperhalusi oleh RSM dan didapati menjadi 12.25 jam, 35 ºC dan 6.5, memberikan hasil xylitol maksimum dan produktiviti sebanyak 86.57% dan 1.33 g/L•h,. Pengoptimuman keadaan proses yang menggunakan strategi berurutan menyebabkan peningkatan 1.55 kali ganda dalam sintesis xylitol secara keseluruhan. Kajian ini dibangunkan dengan pendekatan medium baru untuk meningkatkan pengeluaran xylitol dengan menggunakan MWSHH.

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ºC	Degree Celsius
E^{mM}	Millimolar extinction coefficient
g	Gram
µg	Microgram
h	Hour
kDa	Kilodalton
kg	Kilogram
k_m	Michaelis-Menten constant (mM)
L	Liter
mL	Milliliter
µL	Microliter
min	Minute
mM	Millimolar
µM	Micromolar
nm	Nanometre
%	Percentage
Q_p	Volumetric productivity (g/L·h)
R	Correlation coefficient
R^2	Determination coefficient
rpm	Revolutions per minute
sec	Second
V_{max}	Maximum reaction rate/velocity (µM/min)
$Y_{p/s}$	Xylitol yield factor (g/g)

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CCD	Central composite design
CFE	Cell free extract
DCW	Dry cell weight
DP	Degree of polymerization
dsXR	Dual specific xylose reductase
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetate
EFS	Extractive-free sample
FCR	Folin-Ciocalteu reagent
FCW	Fresh cell weight
FFD	Fractional factorial design
G6PD	Glucose 6-phosphate dehydrogenase
GATT	General Agreement on Tariffs and Trade
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IER	Ion exchange resin
LCM	Lignocellulosic material
LDL	Low density lipoprotein
LDPs	Lignin degradation products
LSR	Liquid to solid ratio

MNRE	Ministry of Natural Resources and Environment
msXR	Monospecific xylose reductase
MWS	*Meranti* wood sawdust
MWSHH	*Meranti* wood sawdust hemicellulosic hydrolysate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OD	Optical density
o.d.b.	Oven dry basis
OFAT	One-factor-at-a-time
PC	Potential concentration
PMSF	Phenylmethylsulfonyl fluoride
RSM	Response surface methodology
SD	Standard deviation
TAPPI	Technical Association of Pulp and Paper Industry
UNCTAD	United Nations Conference on Trade and Development
UV-Vis	Ultra violet-visible
WHO	World Health Organization
XOS	Xylooligosaccharide
XR	Xylose reductase
YPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

In most of the bioprocesses, a large part of the resource utilizations and manufacturing costs are involved in product recovery and purification. The integration of bioconversion and *in situ* product removal can improve the yield and thereby reduce the production costs. A highly selective bioprocess can be developed employing lignocellulosic material (LCM) with a biocatalyst. A wide diversity of LCMs in nature can make the process highly flexible and generic.

The functional sweetener industries are receiving a rapid growth due to the increasing consumer demand for sugar-free and low calorie products. The sweeteners receiving this high priority are the sugar alcohols such as xylitol. Xylitol is a sugar alcohol having sweetening power similar to that of sucrose. It is not only used as a sugar-free sweetener but also has unique characteristics that find applications in food, healthcare, pharmaceutical, and cosmetical industries (Roberto et al., 1995). The most significant application of xylitol is as an ideal sweetener for diabetic patients because of its insulin independent metabolism (Pepper and Olinger, 1988 and Ylikahri, 1979). Other potential uses of xylitol are: as an anticariogenic agent in toothpaste formulations, as thin coatings on vitamin tablets, in chewing gum, candy, ice cream, mouthwashes, beverages and in bakery products (Emodi, 1978; Hyvönen and Koivistoinen, 1982 and Mäkinen, 1992). Xylitol is naturally present in many fruits and vegetables, but its extraction from these sources would not be economically feasible due to the high-cost and relatively low xylitol content of the raw materials (Chen et al., 2010; Parajó et al.,

1998a and Winkelhausen and Kuzmanova, 1998). Xylitol is industrially produced by catalytic reduction of pure D-xylose and can also be produced by biotechnological approaches. The current technology for commercial xylitol manufacturing is based on chemical hydrogenation of pure xylose in the presence of a nickel catalyst at high temperature and pressure. The resulting xylitol is purified and recovered by chromatographic method (Melaja and Hämäläinen, 1977 and Ojamo et al., 2009). This technique of xylitol recovery is extensive and costly and thus the final product is more expensive than other polyols (Nigam and Singh, 1995). In view of alternatives to the conventional process, two biotechnological approaches seem promising: the microbial process and the enzymatic approach.

The microbial process is being examined extensively as an alternative to the chemical process. This process uses bacteria, fungi, and yeast for xylitol production from pure xylose or hemicellulosic hydrolysate. Yeasts are considered as the best xylitol producers among the microorganisms (Nigam and Singh, 1995). In the microbial process, cell recycling requires membrane filtration that makes it an unattractive method for large-scale production of xylitol due to high membrane fouling problems (Granström et al., 2007a). In addition, xylitol recovery from fermented broth is still a great bottleneck, and there is no method available that allows an efficient purification and recovery of xylitol, which is necessary for xylitol production to become economically viable (Mussatto and Roberto, 2005). The benefit of the microbial process over chemical procedures is its lower cost due to the non-necessity of extensive xylose purification (Parajó et al., 1998a). However, the microbial method has not yet been able to accumulate the advantages of the chemical process. The synthesis of xylitol from xylose by enzyme technology can be an attractive alternative to both chemical and microbial processes. Compared to the microbial process, the enzymatic approach to xylitol synthesis is expected to achieve a substantial increase in productivity as mass transfer limitations are avoided in an enzyme reactor. One of the most significant advantages of *in vitro* enzyme-based xylitol production is that it can afford an easy recovery of xylitol. There are scarce reports on the enzymatic conversion of pure xylose to xylitol using isolated xylose reductase (XR) from yeast (Kitpreechavanich et al., 1984; Nidetzky et al., 1996 and Park et al., 2005).

As xylitol is a high value product with rapid growing market potential, research for alternative strategies on its production is essential. This study find out one of the alternatives that are bioconversion of cheap *Meranti* wood sawdust (MWS) biomass, which needs hydrolysis followed by enzymatic (isolated XR) conversion of xylose in the hydrolysate to xylitol. The abundance of renewable wood sawdust offers an advantage to obtain xylose as a substrate to produce xylitol. An efficient hydrolysis of MWS hemicellulose into its sugar monomers is a key step in producing xylitol in a cost-effective and environmental friendly process. This enzymatic process will be an attractive and promising alternative approach to the chemical process.

1.2 PROBLEM STATEMENT

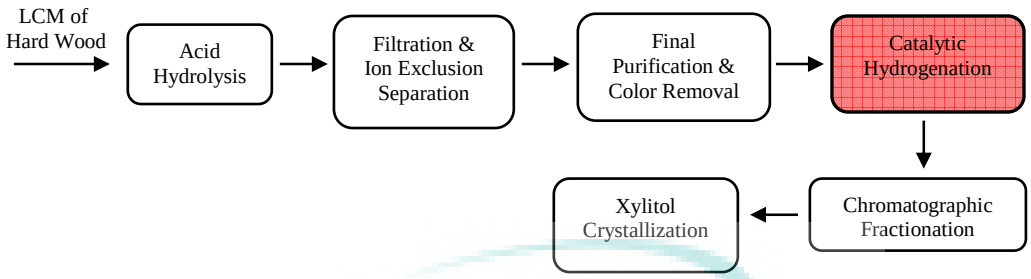
Xylitol can be produced by catalytic reduction of pure D-xylose or biological approaches. The production of xylitol through a chemical process is highly expensive due to difficult separation and purification steps. On the other hand, microbial process on an industrial-scale is not feasible due to decreased yield or productivity. So far, however, there is little research regarding applications of the novel enzyme technology for xylitol synthesis. All experiments in the enzymatic approach have only been carried out using high priced commercial xylose as the substrate. There are no reports on the enzymatic production of xylitol from xylose present in the lignocellulosic biomass. The enzymatic synthesis of xylitol from lignocellulosics is an attractive and promising alternative to the chemical process. Thus, some of the research problems that arise in xylitol production can be stated as follows:

Xylitol is currently manufactured in industrial-scale by chemical hydrogenation of pure xylose obtained from hardwood in the presence of a nickel catalyst at elevated temperature and pressure. The conventional chemical process requires various separation and purification steps to obtain pure xylose that can only be utilized for catalytic reduction (Granström et. al., 2007a and Melaja and Hämäläinen, 1977). The recovery of xylitol is only about 50–60% of the xylan fraction or 8–15% of the initial raw material, and the resultant product becomes very costly because of the extensive purification procedures (Kamal et al., 2011; Nigam and Singh, 1995 and Saha, 2003). The xylitol market is growing very fast and at present is estimated to be US$ 340

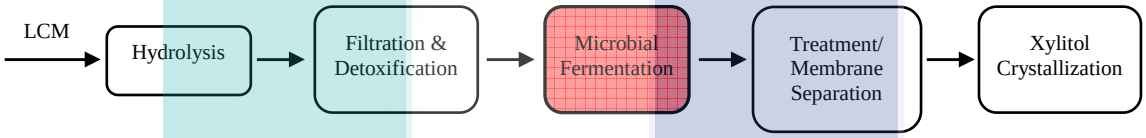
million per year and priced at US$ 6–7 per kg (Povelainen, 2008 and Zhang et al., 2012). The major drawbacks to this chemical process are: requirement of high temperature (80–140 ºC) and pressure (up to 50 atmosphere), use of expensive catalyst, needed extensive separation and purification steps to remove byproducts derived mainly from the hemicellulosic hydrolysate (Saha, 2003), and disposal of nickel contaminated wastewater (Hwan et al., 2003). Hence, the chemical process is laborious, cost- and energy-intensive.

In the microbiological process, yeasts have been studied extensively in the past few decades (Barbosa et al., 1988; Guo et al., 2006; Nigam and Singh, 1995; Onishi and Suzuki, 1969; Sampaio et al., 2008 and Zhang et al., 2012). Although the yield of xylitol from microbiological reduction of xylose could be enhanced (from 65–85% and 86–100%) by applying different production methods using wild-type and recombinant yeasts, the chemical process would still be very competitive in terms of industrial-scale production. The synthesis of xylitol through the microbial fermentation is limited by certain factors such as precise control of culture conditions (temperature, pH, shaking, aeration, cell inhibitors, etc.), expensive nutrients, huge water requirement, and the type of process (Tomotani et al., 2009). Thus, the application of the microbial process on an industrial level is time-consuming, being associated with some preparatory activities such as sterilization and regular inoculum development involving high input of energy, labor, and time, leading to decreased productivity. It is, therefore, necessary and important to explore alternative methods for the effective production of xylitol. Figure 1.1 shows a comparison of conventional, microbial and enzymatic process for xylitol production. Xylitol is a functional food product that has attracted global demand due to its potential application in food, health, pharmaceutical, and cosmetical sectors. Unfortunately, xylitol is imported because Malaysia has not yet developed its own technology to manufacture it. Despite its broad application, the use of xylitol as sweetener is limited by its high price (Parajó et al., 1998a). The cost of xylitol has risen as the hardwood chips used in its manufacturing process have become increasingly scarce. The high cost involved in large-scale production of xylitol seems responsible for its limited commercial use. The aforementioned reasons have inspired the author to work toward the development of improved technologies to lower the production costs of xylitol.

(a) Conventional (chemical) process



(b) Microbial process



(c) Enzymatic approach

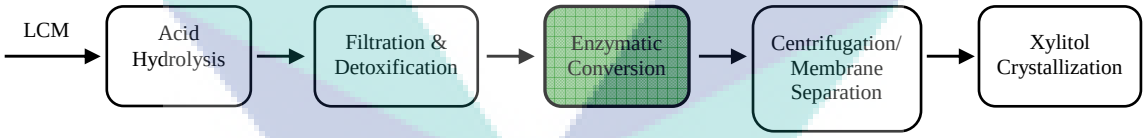


Figure 1.1: Schematic representation of (a) conventional, (b) microbial and (c) enzymatic approach of xylitol production

It was reported that sugar (sucrose) can cause hypoglycemia and weight gain leading to diabetes and obesity in both children and adult humans. It raises blood pressure, triglyceride and bad cholesterol (LDL) levels, and increases the risk of heart disease. It also causes tooth decay and periodontal (gum) disease leading to tooth loss and systemic infections. Tooth decay and periodontal disease are serious problems all over the world, especially in Malaysia. According to the World Health Organization (WHO) in 2005, periodontal disease affected 10–15% of the adult population worldwide (Petersen and Ogawa, 2005). Diabetes mellitus is another common disease causing significant mortality and morbidity. Worldwide, the number of people with diabetes was estimated to be 135 million (4.0%) in 1995; in 2000, it was 154 million,

and in the year 2025, it is expected to cross 300 million (5.4%) with the main increase being in the developing countries. The projected increase in diabetic people in the developed countries is 42%, but in the developing countries, the increase is estimated to be 170%. In Malaysia, the total number of people with diabetes mellitus was reported to be 6.3% in 1986 and in 1996; the prevalence had risen to 8.2%. The WHO has reported that in 2030, Malaysia would have a total number of 2.48 million diabetics compared to 0.94 million in 2000 that is a 164% increase (King et al., 1998 and Mafauzy, 2006). Xylitol is one of most suitable and crucial alternatives to conventional sugar due to its beneficial health effects (Granström et al., 2004, 2007b). Hence, a better xylitol production method needs to be adopted. One of the most attractive procedures is enzymatic production. This study was conducted to explore an alterative approach where hemicellulosic hydrolysate could be utilized as a source of xylose to synthesize xylitol by xylose reductase (XR) with the following objectives.

1.3 RESEARCH OBJECTIVES

The overall objective of this research was to synthesize xylitol from *Meranti* wood sawdust hemicellulosic hydrolysate (MWSHH) by locally prepared XR enzyme. The specific objectives of this study are listed below:

i. To study the kinetics of acid hydrolysis of MWS by H_2SO_4 for the production of xylose, glucose, furfural, and acetic acid and to develop kinetic models for predicting their optimum values.

ii. To optimize MWS acid hydrolysis conditions in order to maximize xylose recovery in the hemicellulosic hydrolysate with low concentration of undesired products and to develop mathematical model.

iii. To prepare and characterize xylose reductase (XR) from yeast *Candida tropicalis* to be used in subsequent xylitol bioproduction.

iv. To determine kinetic parameters of XR, K_m and V_{max}, in order to investigate their effect on xylitol production in batch system.

v. To optimize the key operating conditions that influence xylitol bioproduction from MWSHH by employing a sequential optimization strategy based on OFAT and experimental designs, and to develop mathematical models for predicting optimal yield and productivity of xylitol.

1.4 OVERALL RESEARCH FRAMEWORK

This study concentrates on the enzymatic approach for synthesizing xylitol from MWSHH by XR. Therefore, this research will focus on four major phases: MWS biomass characterization, xylose recovery, XR preparation, and xylitol production. A framework of this research is outlined in Figure 1.2.

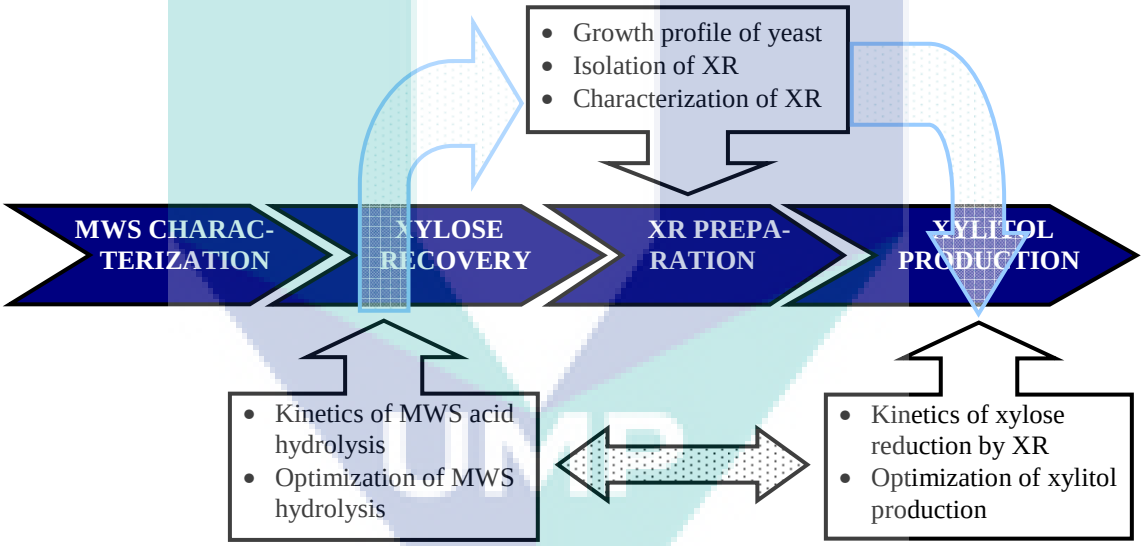


Figure 1.2: A framework of the present research

1.5 SCOPE OF THE STUDY

For achieving the stated objectives, the following scopes of the research have been identified and performed:

i. *Meranti* wood sawdust (MWS) was collected from local sawmill and sun dried. The main structural components of MWS were determined by standard methods.

ii. Kinetic studies on sulfuric acid hydrolysis of MWS were conducted according to the Saeman's model (Saeman, 1945). Kinetic and statistical parameters of components generated during hydrolysis of MWS were determined by non-linear regression analyses.

iii. The effective ranges of process factors (residence time, temperature, H_2SO_4 concentration, and liquid to solid ratio) were selected through one-factor-at-a-time (OFAT) method during hydrolysis of MWS. The hydrolysis conditions were optimized with response surface methodology (RSM).

iv. Sugars (xylose, glucose and arabinose), acetic acid, furfural, and hydroxymethylfurfural (HMF) concentrations in the hemicellulosic hydrolysate were measured by HPLC. Total contents of lignin degradation products (LDPs) were estimated using UV-Vis spectrophotometer.

v. The yeast *Candida tropicalis* was adapted by sequentially transferring and growing cells in media containing increasing concentrations of MWS hemicellulosic hydrolysate (MWSHH; 10–100% (v/v)) supplemented with nutrients. Growth profile of adapted *C. tropicalis* was studied.

vi. XR enzyme was locally prepared from adapted yeast strain by sonication. The enzyme activity, stability, and protein content were measured by UV-Vis spectroscopy.

vii. The kinetic parameters (K_m, V_{max}) of XR were determined following the Michealis-Menten and Lineweaver-Burk method (Lineweaver and Burk, 1934). The inhibitory effects of selected byproducts in the MWSHH on XR activity were assessed and the minimum inhibitory concentration (MIC) was determined.

viii. The effects of reaction variables (reaction time, temperature, pH, xylose, NADPH and enzyme concentration, and agitation rate) on xylitol production were examined by OFAT strategy. The significant variables were screened using fractional factorial design (FFD), and xylitol production was optimized by RSM.

ix. Xylitol solution was analyzed via HPLC and UV-Vis spectroscopy.

1.6 SIGNIFICANCE OF THE STUDY

There is a growing demand for bulk sugar substitutes that are suitable for diabetics and having anticariogenic properties. In this field, xylitol is one of the most promising sweeteners, but the volume of use is small because of its cost and lack of availability. The conventional chemical synthesis of xylitol synthesis is very costly. The fermentative process is also not feasible at industrial level because of low productivity as well as downstream processing problem. The enzymatic approach for xylitol production would be safe and environmental friendly. This approach can overcome the bottlenecks of the conventional process that is exclusively used at present and also the fermentation process that is still under investigation. This study was carried out to produce xylitol from MWS by locally prepared XR. The details of the significance of the present work are stated below:

i. MWS is available at very low cost throughout the year in Malaysia, and the economical disposal of them is a serious problem to the wood industries. Its hydrolysis to yield xylose could be a good alternative to manage this abundant waste. Furthermore, the utilization of MWS as raw material to produce xylose and/or xylitol has a dual consequence: the utilization of waste and the generation of high value product that would be helpful for environmental management and economic development.

ii. Despite a wide range of biotechnological applications, XR is not available commercially probably due to its high production cost. The use of high priced xylose in the growth media probably limited the large-scale production of XR from xylose-fermenting microbes for its industrial application. The cost of XR preparation could be reduced if hemicellulosic hydrolysate of cheap MWS could be used as a source of xylose. Hence, the utilization of MWSHH as a source of xylose will provide an industrially important enzyme XR from a sawmill waste.

iii. The enzymatic synthesis of xylitol from xylose of MWS is an attractive alternative to the chemical process that might accumulate the major advantages offered by conventional process. This approach might have great potential since it is a simple process with high substrate specificity, high yield, and low energy requirements. This study will serve as a benchmark for further work on enzyme-based *in vitro* production of xylitol from LCMs.

iv. The enzymatic method might reduce the production costs and improve productivity of xylitol by using cheap MWS as raw material, eliminating idle time required for cleaning and sterilization, reducing labor cost, steady product synthesis, simple process control, and higher purity with no other chemical than enzyme.

v. The acid hydrolysis of MWS can also yield a valuable solid residue mainly composed of cellulose and lignin (i.e., cellulignin). This solid residue can be utilized in the production of glucose solutions for the generation of ethanol, sorbitol, lactic acid, or in pulp processing for making high grade paper.

1.7 THESIS OUTLINE

The thesis consists of seven chapters. It begins with Chapter 1 (Introduction), where research background, problem statement, and research objectives are focused. In this chapter a research roadmap, scope and significance of the study are also highlighted. Chapter 2 (Literature review) describes the literature on the various methods applied for xylitol production, LCM hydrolysis and hydrolysate treatment that

are relevant to this work. A brief description about the modeling via statistical program is also covered. Chapter 3 (Materials and methods) presents the detail of the materials and chemicals utilized in this work. In this chapter, the overall experimental flowchart and operational framework are given. A detailed experimental procedures and analytical methods for MWS characterization, xylose recovery, XR preparation and xylitol synthesis are given. A description of the parameter design, screening, and optimization approaches are also outlined. Results and discussion are covered by three Chapters (Chapter 4–6). In Chapter 4, the results of characterization of MWS and XR are presented. The parameter designs, kinetic studies on MWS hydrolysis and optimization of parameters for xylose recovery are addressed in Chapter 5 (Results and discussion). The detailed discussion on the mathematical models and optimum conditions obtained by RSM for the recovery of xylose is also addressed. In Chapter 6, design of parameter for xylitol synthesis, screening process and optimization of process parameters are presented. A detailed discussion on the RSM models and optimum conditions achieved for xylitol production is also presented. Finally, general conclusions for all the experiments in this study are highlighted in Chapter 7 (General conclusion and recommendations for future work) along with some recommendations for future work. The thesis is completed with references and appendices.

1.8 CONCLUSION

This chapter introduces the chemical and biotechnological processes of xylitol production. The enzymatic approach, the main focus of this research work, has been extensively elaborated. The disadvantages of chemical and microbial process altogether come up as specific research problems in xylitol synthesis. The chemical process is complicated, and expensive due to difficult separation and purification steps, and the microbial process on an industrial-scale is not feasible due to decreased productivity of xylitol. Thus, better strategies required to overcome the problems are the targets of this study.

CHAPTER 2

LITERATURE REVIEW

A review of the previous studies that are relevant to xylitol production was conducted in this chapter. The different methods of xylitol production are discussed, highlighting the advantages and disadvantages associated to each process for industrial application. The structure, sources, properties and applications of xylitol are documented at the beginning of the chapter. The major steps in the production of xylitol from lignocellulosic material (LCM) such as LCM hydrolysis to extract xylose, detoxification of hydrolysate, and hydrogenation of xylose are also reviewed along with optimization strategies. All of these interested topics are detailed in the following text.

2.1 INTRODUCTION

The prime objective of this chapter is to review the previous literature on the processes involving xylitol production, taking into account the sources and properties of xylitol, lignocellulosic material (LCM) and its hydrolysis, hydrolysate detoxification, chemical and biotechnological methods of xylitol synthesis, microorganisms and their xylose metabolism, xylose reductase (XR) preparation, and optimization strategies. In addition, this chapter tries to identify ways to improve enzymatic xylitol production so that it can compete with the current chemical process. In particular, the main steps in the production of xylitol from LCM, such as hydrolysis of LCM to extract xylose, detoxification of hydrolysate, and hydrogenation of xylose, are reviewed and discussed. Xylitol is a five-carbon sugar alcohol that has widespread potential applications in the food industry, but the volume of use is small because of its cost and lack of availability. Xylitol is industrially produced by catalytic reduction of pure D-xylose but can also be

produced by biotechnological approaches. The hemicellulosic sugar xylose is extracted from LCMs mainly by acidic or enzymatic hydrolysis. Industrially, xylitol is currently manufactured by chemical hydrogenation of pure D-xylose in the presence of a nickel catalyst at high temperature and pressure (Melaja and Hämäläinen, 1977 and Ojamo et al., 2009). The recovery of xylitol is about 50–60% of the xylan fraction or 8–15% of the initial raw material, and the resultant product is very expensive because of the extensive purification procedures (Nigam and Singh, 1995 and Parajó et al., 1998a). With a view to find an alternative to the conventional process, two biotechnological approaches seem promising: the microbial process and the enzymatic approach.

The best xylitol producers among the microorganisms are considered to be yeasts especially, the genus *Candida* (Nigam and Singh, 1995; Winkelhausen and Kuzmanova, 1998 and Zhang et al., 2012). In the microbial process using wild-type and recombinant yeast, the yield of xylitol obtainable from D-xylose is in a range of 65–85% (Nigam and Singh, 1995) and 86–100% of the theoretical value (Bae et al., 2004 and Govinden et al., 2001), respectively. The application of microbial process on an industrial level is time-consuming, being associated with some preparatory activities that lead to decreased productivity (Prakasham et al., 2009). The benefit of the microbial process over chemical procedures is its lower cost due to the non-necessity of extensive xylose purification (Parajó et al., 1998a). However, the microbial method does not yet have the advantages of the chemical process because of the low productivity of xylitol and downstream processing problems.

Xylitol production from xylose by enzyme technology can be an attractive alternative to both chemical and microbial processes. There are scarce reports regarding the enzymatic conversion of commercial pure xylose to xylitol using isolated XR from yeast (Kitpreechavanich et al., 1984; Neuhauser et al., 1998 and Nidetzky et al., 1996). The bioconversion of D-xylose into xylitol is above 95% by the NADH-dependent reduction of xylose using XR (Nidetzky et al., 1996). The high cost involved in large-scale production of xylitol seems responsible for its limited commercial use. This has inspired researchers to work toward the development of improved techniques to reduce the costs of production. In this field, the enzymatic approach to xylitol production from xylose present in the LCM may provide an alternative for the chemical process.

2.2 XYLITOL

Xylitol is a naturally occurring five-carbon sugar alcohol with a molecular formula of $C_5H_{12}O_5$ and is used commercially as a natural sweetener in various food products. The chemical structure of xylitol is shown in Figure 2.1. It has the sweetness and caloric content equivalent to sucrose (4 cal/g) and thus has the great potential to replace sucrose in low-calorie products (Heikkilä et al., 1992).

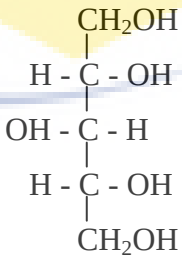


Figure 2.1: Chemical structure of xylitol

2.2.1 Natural Sources of Xylitol

Xylitol is naturally found in small amounts, in many fruits and vegetables (Table 2.1), as well as in yeasts, lichens, seaweeds and mushrooms, among which the yellow plum has the highest amount (almost 1% on a dry solid basis) (Affleck, 2000; Parajó et al., 1998a and Winkelhausen and Kuzmanova, 1998). It is also a normal metabolic intermediate produced during mammalian carbohydrate metabolism at a range of 5–15 g per day in an adult human (Winkelhausen and Kuzmanova, 1998). Xylitol extraction from its natural sources is impractical and economically not feasible due to the relatively low xylitol content of the raw materials and high production costs (Chen et al., 2010 and Parajó et al., 1996).

2.2.2 Properties of Xylitol

The technological properties of xylitol as food are similar to those traditionally expected for bulk sweeteners. For example, (i) it dissolves readily in water; (ii) its caloric value is similar to those of other sugar alcohols; and (iii) its relative sweetness is

about the same as sucrose, being 2–2.5-times higher than sorbitol or mannitol (Emodi, 1978). Xylitol is thermochemically stable, and does not undergo Maillard reactions and caramelization because of the absence of aldo- and keto-groups. Its solutions are less viscous than those of sucrose. Another notable property is its endothermic dissolution, with a heat of solution more than eight times that of sucrose and almost double of sorbitol. After long-term storage, products containing xylitol showed colour and taste properties superior to products containing other sweeteners (Parajó et al., 1996). Xylitol does not have any unpleasant aftertaste like other sugar substitutes from a taste perspective (Heikkilä et al., 1992). The glycaemic index (a measure that indicates how quickly foods enter the bloodstream) of sugar is rated at 100 and xylitol at only 7.0 (Natah et al., 1997). The physical properties of xylitol are given in Table 2.2.

Table 2.1: Natural sources of xylitol

Product	Xylitol (mg/100 g dry substance)
Banana	21
Raspberry	268
Strawberry	362
Yellow plum	935
Carrot	86.5
Endive	258
Onion	89
Lettuce	131
Cauliflower	300
Pumpkin	96.5
Spinach	107
Kohlrabi	94
Eggplant	180
Leek	53
Fennel	92
White mushroom	128
Brewer's yeast	4.5
Chestnut	14
Carrot juice	12
Lamb's lettuce	273

Source: Affleck, R.P. (2000)

Table 2.2: Properties of xylitol

Property	Xylitol
Formula	$C_5H_{12}O_5$
Molecular weight	152.15
Appearance	White, crystalline powder
Odor	None
Solubility at 20 ºC	169 g/100 g H_2O
pH in water (1 g/10 mL)	5–7
Melting point (ºC)	93–94.5
Boiling point (at 760 mmHg)	216 ºC
Density (bulk density) (15 ºC)	1.50 g/L
Caloric value	4.06 cal/g (16.88 J/g)
Moisture absorption (%) (4 days, 20–22 ºC)	
at 60% relative humidity	0.05
at 92% relative humidity	90
Density (specific gravity) of aqueous solution (20 ºC)	
10%	1.03
60%	1.23
Heat of solution, endothermic	36.61 cal/g (153.76 J/g)
Viscosity (cP) (20 ºC)	
10%	1.23
40%	4.18
50%	8.04
60%	20.63

Source: Affleck, R.P. (2000)

2.2.3 Application of Xylitol

There is an increasing commercial demand for xylitol as a bulk sugar substitute, being suitable for diabetics and as non-cariogenic. A large number of investigations have shown the useful effects of xylitol as a sweetener when employed alone or formulated in combination with other sugars (Hayes, 2001; Jannesson et al., 2002; Lynch and Milgrom, 2003; Mäkinen, 2000a, b and Pepper and Olinger, 1988). Xylitol has found its potential applications at least in three industries, namely food, odontological and pharmaceutical (Prakasham et al., 2009 and Roberto et al., 2003). Xylitol has been shown almost equally effective in foods, chewing gum, soluble dragees or related products as well as in toothpaste (Rao et al., 2008). Due to the secondary

gastrointestinal impacts resulting from the slow absorption rate of xylitol, the maximum allowable amount ingested is 20 g/dose or 60 g/day (Parajó et al., 1998a). Xylitol has attracted global demand mainly due to its insulin-independent metabolism, anticariogenecity, sweetening power equivalent to sucrose, and pharmacological properties. The interest in xylitol production originated from its applicability as a diabetic sweetener (Mellinghoff, 1961; Mäkinen, 2000a and Granström et al., 2007b). At present, xylitol is approved for use in foods, pharmaceuticals, cosmeceuticals, and oral health products in more than 50 countries. Its market is growing very fast and is currently estimated to be over US$ 340 million/year and priced at US$ 6–7 per kg (Povelainen, 2008 and Zhang et al., 2012). The important applications of xylitol are detailed below.

Xylitol in Food Industries

Xylitol has been used as a food ingredient and sweetening agent for over 40 years and its properties have been extensively studied. It has widespread applications in almost all sectors of the food industry such as confectionery, chewing gum, hard coating applications, chocolate, dairy products, baked goods (Povelainen, 2008), hard candy, toffee, ice cream, yoghourt, and drinks (Parajó et al., 1996). Xylitol is applied either as the sole sweetener or in conjunction with other sweeteners in the preparation of a variety of reduced-energy or sugarless confectionery products suitable for infants and diabetics. Bakery products, spices and relishes, jams, jellies, marmalades and desserts represent other potential sectors for the applications of xylitol as a sweetener (Winkelhausen and Kuzmanova, 1998). Xylitol has several advantages as a food ingredient. It does not show Maillard reaction, which is responsible for both darkening and reduction in the nutritional value of proteins; when continuously supplied in the diet, it limits the tendency to obesity, and the incorporation of xylitol in food formulations improves the color and taste of preparations without causing undesired changes in properties during storage. Extensive studies on dietary and technological characteristics of xylitol have proved it to be a beneficial sweetener when used in jams, yoghurts (providing texture, color and taste, stable for longer periods than those of products formulated with traditional sugars such as sucrose) or frozen desserts (Hyvönen and Slotte, 1983 and Parajó et al., 1998a).

Xylitol in Pharmaceutical Industries

Xylitol is an interesting product for the pharmaceutical industry owing to its tooth friendly nature, capability of preventing otitis, ear and upper respiratory tract infections and its possibility of being used as a sweetener or excipient in syrups, tonics and vitamin formulations. Moreover, xylitol helps in controlling hyperglycemia as its metabolism is independent of insulin (Prakasham et al., 2009 and Yoshitake et al., 1973). Xylitol generates a feeling of vaporization in the nasal and oral cavities due to its negative heat of dissolution (Parajó et al., 1998a). It is utilized as a constituent of coating material for pharmaceutical products such as vitamin or expectorant, generally in combination with sorbitol, mannitol, and adipic or citric acids (Pepper and Olinger, 1988), and in the formulation of dietary complements (e.g., vitamins, amino acids, trace elements and nonreducing sugars) (Parajó et al., 1998a). Clinical and field studies have shown that xylitol could prevent ear infections in small children when administered in chewing gum (Uhari et al., 1996). Xylitol can replace antibiotics in the treatment of acute medium otitis (Canilha et al., 2004). In addition, xylitol possesses laxative properties. It helps in gingivitis reduction and in halitosis control (Martínez et al., 2007).

Xylitol in Odontological and Oral Health Industries

Xylitol is widely used in the odontological industry due to its anticariogenicity (Hyvönen and Koivistoinen, 1982), tooth rehardening and remineralization properties. The main application is for the prevention of dental caries because xylitol inhibits growth of organisms responsible for tooth decay (Mäkinen, 2000a, b). It prevents both osteoporosis (Mattila et al., 2002) and formation of acids that attack the tooth enamel (Shen et al., 2001). For all of these advantages, xylitol has attracted the attention of odontological industry (Canilha et al., 2004). Xylitol is also used in the formulation of personal health care products such as mouthwash, toothpaste, and other oral hygiene products (Affleck, 2000 and Winkelhausen and Kuzmanova, 1998).

Generally, it is accepted, that the consumption of sugars and other fermentable carbohydrates in diets is one of the major factors for dental caries (Povelainen, 2008). Xylitol and other polyols are usually considered as non-cariogenic, meaning that they

do not contribute to tooth decay and are thus increasingly used as replacements for fermentable carbohydrates in foods. However, it has been shown that xylitol is not only non-cariogenic but also actually prevents tooth decay. The ability to inhibit the development of caries has been demonstrated in numerous clinical and field researches. These researches lead to a conclusion that when xylitol containing confectionary or chewing gum is consumed as part of the normal diet, new caries incidences are typically reduced by 40–80% (Alanen et al., 2000 and Mäkinen et al., 1995). Xylitol is not fermentable and thus cannot be converted to acids by oral bacteria, so it helps to maintain a proper alkaline/acid balance in the mouth. This alkaline condition is inhospitable to all the destructive bacteria, principally the worst variety, *Streptococcus mutans*. Xylitol also inhibits plaque formation (Söderling et al., 1991). In light of the scientific information presently available, xylitol can be considered as the best of all alternative sweeteners with respect to caries prevention.

Xylitol and Diabetes

The most significant application of xylitol is its use as an alternative sweetener in food for diabetic patients (Pepper and Olinger, 1988). Xylitol is slowly absorbed; therefore, when ingested, the rise in blood glucose and insulin response related to the ingestion of glucose is significantly reduced. Xylitol is accepted for diabetic's consumption because it does not need insulin to regulate its metabolism. Furthermore, its metabolism does not involve glucose 6-phosphate dehydrogenase (G6PD), and it is, therefore, an ideal sweetener for G6PD deficient members of the population (Nigam and Singh, 1995). The tolerance of xylitol by diabetic patients lies in the facts that its (i) direct absorption mainly in the liver, and (ii) indirect metabolism in the intestinal tract by intestinal bacteria, are not insulin mediated. Xylitol causes very limited increase in the glucose and insulin levels in blood (Hassinger et al., 1981) when compared with the changes caused by glucose or sucrose (Parajó et al., 1998a and Ylikahri, 1979). These functional properties make xylitol useful for post-operative or post-traumatic states while the excessive secretion of stress hormones (such as catecholamines, cortisol, growth hormone, glucagon, etc.) creates insulin resistance and inhibits the efficient utilization of glucose. Moreover, xylitol does not react with amino acid, allowing its use for parenteral nutrition (Parajó et al., 1998a).

2.3 WOOD SAWDUST

A wide variety of lignocellulosic biomass sources is available for further conversion and utilization. The selection of biomass feedstock bears paramount importance from both techno- and socio-economical points of view. From ethical perspectives, the biomass should not compete with the food chain. Lignocellulosic waste with a low or even negative value, such as agricultural and forest residues are preferred. Furthermore, it is also advantageous to select biomass sources that are locally available with low or no cost throughout the year. Based on these criteria, the wood sawdust could be an excellent biomass feedstock for further bioconversion and utilization and hence has been selected as the raw material of choice for this research.

Malaysia is known for its potential in renewable resources of lignocellulosic biomass such as wood sawdust, oil palm waste, sugarcane bagasse and rice straw. The total area of forests in Malaysia is estimated to be 19.52 million hectares, or 59.5% of the total land area, of which 14.45 million hectares or 44% are permanent reserved forests (MNRE, 2006). Malaysia has been successful in the development of its forestry and forest-based industries in the last few decades. The wood based industries have developed from a primary processing industry (e.g., sawmills and plywood mills) into a diversified value-added industry (Woon and Norini, 2002). Sawdust is a sawmill waste, a lignocellulosic material that is available at low cost throughout the year. Sawdust is produced in huge quantities not only by large sawmills but also by small ones, and the economical disposal of them is a serious problem to the wood industries. Although sawdust is generated in large quantities, it has gained little attention as a marketable commodity. It is commonly used as fuel in manufacturing industries and in local utilities with a relatively low heating value. Other uses of sawdust are: as litter and bedding material in livestock and poultry structures, for the production of fiberboards and paper pulp (Arends and Donkersloot-Shouq, 1985 and Harkin, 1969). Therefore, the use of sawdust as feedstock for the manufacture of different value-added commodities is very much desirable.

Sawdust from red *Meranti* species was chosen as raw material in this study because it is one of the most common and popular hardwood species in Malaysia. In

addition, *Meranti* wood sawdust (MWS) was taken as research material because it is abundantly and easily available as waste and generally disposed in landfill areas as the cheapest way of management. MWS is locally available at surrounding areas of the Universiti Malaysia Pahang especially at the wood industrial area Taman Tas, Kuantan, Pahang. The use of MWS can offer immense opportunities in improving the economic conditions of the sawyers and farmers. Thus, MWS utilization will not only solve the disposal problem but also yield a high value product from wood industry waste.

2.4 LIGNOCELLULOSIC MATERIAL

Lignocellulose is the principal constituent of plant cell walls. All lignocellulosic materials (LCMs) are consisted of three biopolymers cellulose (34–50%), hemicellulose (19–35%), lignin (11–30%) and smaller contents of extractives, ash, pectins and proteins. The content of these constituents can vary according to growth conditions, plant species and age (Kumar et al., 2009 and Prakasham et al., 2009).

2.4.1 Cellulose

Cellulose is the major chemical constituent of LCM and is found in an organized fibrous structure. The chemical structure of a cellulose chain is shown in Figure 2.2. It is a linear homopolysaccharide composed of D-glucose units (5000–10,000 units) linked together by β-(1,4)-glycosidic bonds to form a skeleton (Kumar et al., 2009). The repeating unit of the cellulose molecule consists of two glucose anhydride units, called cellobiose, which constitutes cellulose chains (Balat et al., 2008). The size of a cellulose molecule is generally expressed by its degree of polymerization (DP) (i.e., the number of anhydroglucose units that exist in a single chain) (Ramos, 2003). The cellulose chains in LCMs are linked together by hydrogen bonds. The long cellulose fibers are in turn held together with hemicellulose and lignin through hydrogen-bonding interaction and covalent interaction, respectively (Mosier et al., 2005). Cellulose in LCM is present in both crystalline and amorphous forms. In LCMs, about 50–90% of the cellulose chain is joined laterally by hydrogen bonds to form a crystalline (ordered) structure, whereas the remaining fraction is less ordered and is called amorphous cellulose (Jacobsen and Wyman, 2000 and Kumar et al., 2009). The cellulose fibers provide wood's strength

and comprise about 40–50% (w/w) of dry wood (Balat et al., 2008 and Sinağ et al., 2009). Cellulose is susceptible to enzymatic degradation in its amorphous form. Glucose can be released from cellulose through the action of either acid or enzymes by breaking glycosidic linkages (Kumar et al., 2009). The resulting glucose can be transformed to sorbitol or bioethanol as a fuel additive (Sun and Cheng, 2002).

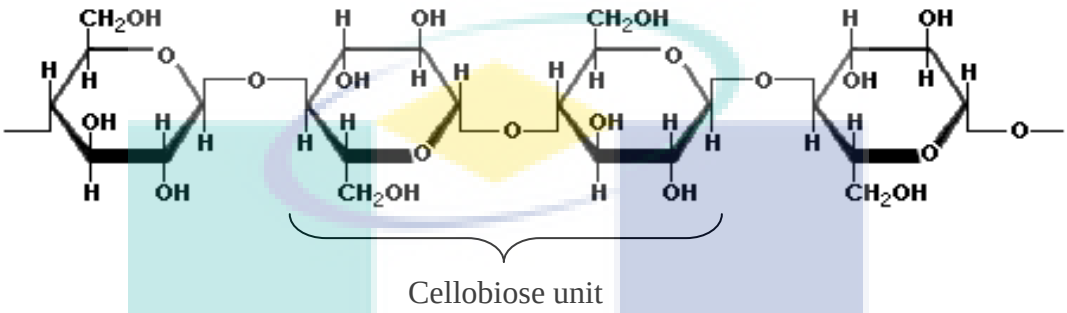


Figure 2.2: Structure of a cellulose chain

2.4.2 Hemicellulose

Hemicellulose, a second major constituent of LCM, is a complex heteropolysaccharide whose chemical composition and structure vary from tissue to tissue within a single plant and from species to species. In general, it is composed of a variety of building block compounds including pentoses (e.g., xylose and arabinose), hexoses (e.g., glucose, galactose, and mannose), and uronic acids (e.g., 4-O-methyl-glucuronic and galacturonic acids) with acetyl side chains. It consists of 100–200 units of pentoses and hexoses in its molecular backbone (Ramos, 2003 and Thomas et al., 2011). Hemicellulose is amorphous in structure, and the variety of branching, linkages, and monomer units contribute to its complex architecture and thereby its variety of conformations and function (Jacobsen and Wyman, 2000). Hemicellulose differs from cellulose in three main aspects. Firstly, it contains several different types of sugar units, whereas cellulose contains only β-(1,4)-D-glucopyranose units. Secondly, it exhibits a considerable degree of chain branching, whereas cellulose is strictly a linear polymer. Thirdly, the DP of cellulose is 10–100 times higher than that of hemicellulose (Thomas et al., 2011). Hemicellulose in LCM is connected to cellulose through hydrogen-bonding interactions and builds a structural matrix (Mosier et al., 2005). This matrix is further bound to lignin to form a lignocellulosic complex (Prakasham et al., 2009).

Hemicellulose normally accounts for 25–35% of the mass of dry wood (28% in softwoods and 35% in hardwoods) (Balat et al., 2008 and Sinağ et al., 2009). Hardwoods (e.g., willow, aspen and oak) and softwoods (e.g., spruce and pine) differ in structure and composition of the hemicellulose. Hemicellulose in softwood has a higher proportion of mannose and glucose units than hardwood hemicellulose, which usually contains a larger proportion of xylose units (Palmqvist and Hahn-Hägerdal, 2000b). During hydrolysis, xylose is the predominant sugar derived from hardwood hemicellulose feedstocks, but arabinose can constitute an appreciable amount of the pentose sugars derived from various agricultural residues and herbaceous crops (such as switchgrass) (Balat et al., 2008). A hardwood hemicellulose consists of O-acetyl-4-O-methylglucuronoxylan and glucomannan. O-acetyl-4-O-methylglucuronoxylan, simply called xylan, is the main hemicellulose in hardwoods. Xylan is found in substantial amounts ranging from 11–35% (dry mass basis) in LCM such as agricultural residues (such as brewer's spent grain, sugarcane bagasse, wheat and rice straw, corncob, coconut or sunflower hulls, and cotton seeds) and hardwoods (Nigam and Singh, 1995 and Parajó et al., 1998a). Xylan hydrolysis produced principally pentose sugar xylose in the hemicellulosic hydrolysates that can be further utilized as a starting substrate to bioproduce xylitol (Mussatto and Roberto, 2008 and Rahman et al., 2006).

2.4.3 Lignin

Lignin is the third significant fraction in LCM and is a polymer of sinapyl-, coniferyl- and coumaryl alcohol units linked by aryl-aryl, alkyl-alkyl, and alkyl-aryl ether linkages. It is found in the plant cell walls, imparting structural support, impermeability, and resistance against microbial attack. In general, softwoods contain more lignin than hardwoods and herbaceous plants such as grasses, agricultural residues (Kumar et al., 2009 and Palmqvist and Hahn-Hägerdal, 2000b). Lignin contents on a dry basis in both hardwoods and softwoods generally range from 20–40% (w/w) and from 10–40% in various herbaceous species such as bagasse, corncobs, straws and rice hulls (Balat et al., 2008). Lignin is amorphous and hydrophobic in nature and is not hydrolyzed by acids, but is soluble in hot alkali, readily oxidized and easily condensable with phenol (Thomas et al., 2011). Cellulose, hemicellulose, and lignin biopolymers are closely associated with each other; thus, biomasses can be regarded as composite

materials in which lignin acts as a protective agent that prevents plant cell destruction by fungi and bacteria for conversion to fuels and chemicals (Prakasham et al., 2009).

2.5 HEMICELLULOSE HYDROLYSIS METHODS

To make the LCM suitable for bioconversion in bioprocesses, cellulose and hemicelluloses must be hydrolyzed into their sugar constituents for use by organisms or enzymes. Hemicellulose is easily hydrolyzable due to its amorphous, branched structure compared to cellulose (Parajó et al., 1998b), which needs severe treatment conditions because of its crystallinity, degree of polymerization, and accessible surface area (Chundawat et al., 2011 and Taherzadeh and Karimi, 2008). The purpose of hydrolysis of the hemicellulosic fraction of LCM, specifically hardwoods and agricultural residues, is to maximize the recovery of pentose sugars (xylose or arabinose) and minimize the coformation of toxic/inhibitory byproducts. Furthermore, hydrolysis of the hemicellulose prepares the cellulosic fraction for subsequent conversion by acid or enzymes and presents particularly promising opportunities for studies that could radically reduce biomass processing costs. Hemicellulose is generally hydrolyzed by either acid, autohydrolysis, or enzymes to produce xylose and/or xylooligosaccharides.

2.5.1 Acid Hydrolysis

Acid hydrolysis is a rapid and simple method for lignocellulosic biomass. The hydrolysis conditions vary with raw material type, acid type and concentration, reaction temperature and time (Pessoa Jr et al., 1996; Prakasham et al., 2009 and Sun and Cheng, 2002). Xylose is the most abundant sugar released in the hydrolysate with a small amount of other sugars while fully grown or aged hardwoods or agricultural residues are utilized as feedstocks. Different acids such as sulfuric (Liu et al., 2012; Mussatto and Roberto, 2005; Rahman et al., 2007 and Romero et al., 2010), hydrochloric (Herrera et al., 2004 and Lavarack et al., 2002), phosphoric (Lenihan et al., 2010 and Vázquez et al., 2007), nitric (Brink, 1993), and acetic (Conner and Lorenz, 1986) acids are commonly employed as catalysts in the hydrolysis process. Acid hydrolysis is mainly of two types based on the concentration of the acid applied: concentrated acid and dilute acid hydrolysis. The major decomposition pathway during acid-catalyzed hydrolysis of LCM is shown in Figure 2.3.

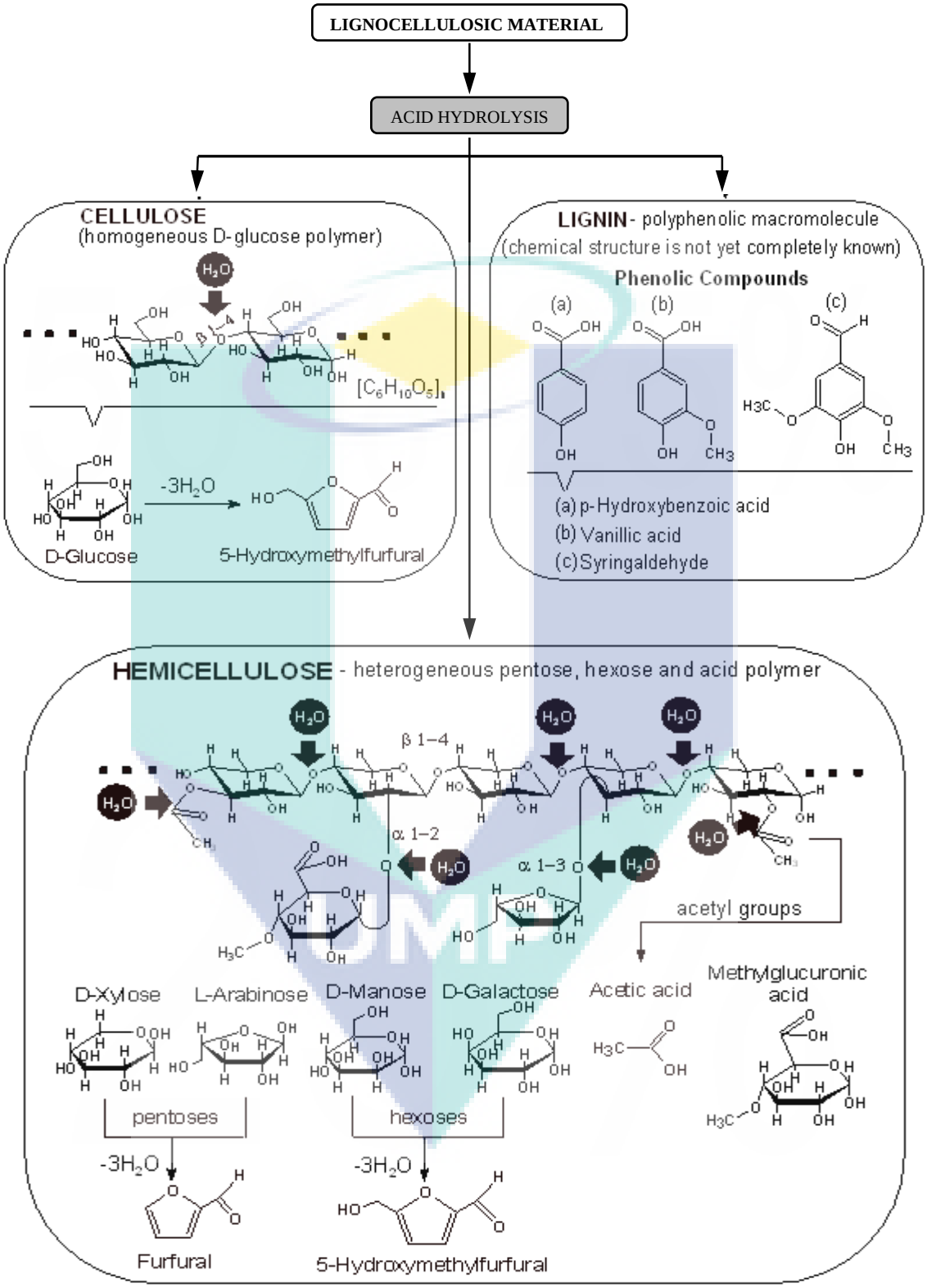


Figure 2.3: Degradation of lignocellulosic material by acid hydrolysis

Source: Marton et al. (2006)

Concentrated Acid Hydrolysis

Concentrated acid hydrolysis is a relatively old method. It is conducted at low operating temperature (<50 ºC) and atmospheric pressure with high acid concentration (50–70%, v/v). Approximately 100% cellulose is converted to glucose in this process (Kumar et al., 2009). Normally, concentrated acids such as H_2SO_4 and HCl have been used to treat LCM. Although the concentrated acids are powerful agents for cellulose hydrolysis, they are toxic, corrosive and hazardous and thus require special reactors that are resistant to corrosion, which makes the hydrolysis process very expensive. Furthermore, the concentrated acid must be recovered after hydrolysis in order to make the process economically feasible (Kumar et al., 2009 and Sun and Cheng, 2002).

Dilute Acid Hydrolysis

Dilute acid hydrolysis is one of the most studied and widely used hydrolysis methods because it is effective and inexpensive (Balat et al., 2008; Liu et al., 2012 and Sun and Cheng, 2005). This method can be applied either as a pretreatment step preceding enzymatic hydrolysis or as the main method of hydrolysis. Dilute acid hydrolysis is generally conducted at elevated temperatures (160–230 ºC) and pressures (~10 atm) (Iranmahboob et al., 2002). The concentration of mineral acids such as H_2SO_4 or HCl used in this hydrolysis process is in the range of 2–5% (Kumar et al., 2009). The dilute H_2SO_4 can effectively hydrolyze hemicellulose into monomeric sugars (xylose, arabinose, glucose, galactose, and mannose) and soluble oligomers. Compared to other hydrolysis methods, it is especially useful for the conversion of xylan in hemicellulose to xylose, which can be further fermented to xylitol or ethanol by many microbial strains (Sun and Cheng, 2005). Dilute acid-catalyzed hydrolytic reaction is very complex, mainly due to the fact that the substrate is in a solid phase and the catalyst in a liquid phase. It is a multistep reaction that takes place sequentially as follows (Herrera et al., 2003): (i) protons diffusion in the wet lignocellulosic matrix; (ii) protonation of a glycosidic bond oxygen that connects monomeric sugars; (iii) cleavage of the gylcosidic linkage, which is considered as the rate-limiting step; (iv) formation of a carbocation as intermediate complex; (v) solvation of the carbocation with water; (vi) regeneration of the proton with cogeneration of the sugar monomer, oligomer or polymer depending on

the position of the glycosidic bond; (vii) diffusion of the reaction products into the liquid phase, and (viii) return of the protons to the second step to begin the cycle again. The initial and the rate-limiting steps are influenced by acid concentration, reaction time, temperature, and liquid to solid ratio (Liaw et al., 2008 and Rao et al., 2006). It is important to choose less severe conditions that will maximize xylose yield while minimizing the formation of byproducts such as acetic acid, furfural, hydroxymethylfurfural (HMF), and lignin degradation products (LDPs). Table 2.3 presents the reported optimum conditions of acid hydrolysis of various LCMs.

Toxic Compounds in Hydrolysate

Acid treatment of MWS is required to produce xylose-rich hemicellulosic hydrolysate which is used as reaction media for subsequent microbial/enzymatic xylitol synthesis. A wide variety of compounds that are toxic to organisms are normally released or formed during acid hydrolysis of LCM (Figure 2.3). The nature of inhibitory components and their concentrations in hemicellulosic hydrolysate depend on both the type of raw material and the hydrolysis condition used (Canilha et al., 2004 and Mussatto and Roberto, 2004). These compounds are usually divided into four main groups based on their origin: furan derivatives, weak acids, phenolic compounds, and heavy metals like chromium, copper, iron, and nickel released from the hydrolysis equipment. Furan derivatives such as furfural and hydroxymethylfurfural (HMF) generated from the degradation of pentoses and hexoses, respectively. Acetic acid, the major weak acid present in the hydrolysate, results from the deacetylation of hemicellulose. Phenolic and other aromatic compounds are derived from the partial degradation of lignin (Marton et al., 2006; Palmqvist and Hahn-Hägerdal, 2000a and Villarreal et al., 2006). HMF can be further decomposed to levulinic and formic acid, the latter also produced when furfural is broken down. Normally, HMF is formed in less amounts compared to furfural by the decomposition of hexose sugars mainly as the small contents of hexoses in hemicelluloses. In addition, the conditions applied in the hydrolysis of hemicellulosic materials do not break down hexoses in large quantities (Palmqvist and Hahn-Hägerdal, 2000b). Other toxic compounds such as resin, terpene, vanillic, syringic, tannic, caprylic, caproic, palmitic, and pelargonic acids are also reported to be formed during chemical hydrolysis of LCM (Bower et al., 2008).

Table 2.3: Reported data on the acid hydrolysis of different lignocellulosic materials

Lignocellulosic materials	Optimum hydrolysis conditions	Products (g/L)	Reference
Eucalyptus globulus (particle size <1 mm)	3% (w/w) H_2SO_4, 130 ºC, 60 min, 8:1 w/w LSR (in autoclave)	18 xylose 3.6 glucose 0.6 arabinose 5.2 acetic acid <0.5 furfural	Parajó et al., 1996
Wheat straw (particle size <1 mm)	1.85% (w/v) H_2SO_4, 90 ºC, 18 h, 20:1 w/w LSR (in reactor)	12.80 xylose 1.70 glucose 2.60 arabinose 0.15 furfural 2.70 acetic acid	Nigam, 2001
Sorghum straw (particle size <0.5 mm)	6% (w/w) HCl, 122 ºC, 70 min, 10:1 w/w LSR (in autoclave)	16.20 xylose 3.80 glucose 2.0 furfural 1.90 acetic acid	Herrera et al., 2003
Brewer's spent grain (particle size <0.5 mm)	3% (w/w) H_2SO_4, 130 ºC, 15 min, 8:1 w/w LSR (in autoclave)	26.70 xylose 4.0 glucose 12.80 arabinose 1.50 acetic acid 0.29 furfural 0.02 HMF 0.91 LDPs	Carvalheiro et al., 2004a
Oil palm empty fruit bunch (particle size <1 mm)	6% H_2SO_4, 120 ºC, 15 min, 8:1 w/w LSR (batch process)	29.40 xylose 2.34 glucose 1.25 acetic acid 0.87 furfural	Rahman et al., 2006
Eucalyptus grandis (particle size <0.5 mm)	0.65% H_2SO_4, 157 ºC, 20 min, 8.6:1 w/w LSR (1.4 L pilot-scale reactor)	13.65 xylose 1.65 glucose 1.55 arabinose 3.10 acetic acid 1.23 furfural 0.20 HMF	Canettieri et al., 2007
Rice straw (particle size 10 mm)	2% H_2SO_4, 126 ºC, 60 min, 10:1 v/w LSR (pressure cooker)	13.30 xylose 2.50 glucose 2.30 arabinose	Liaw et al., 2008

The inhibition intensity is directly associated with the initial concentrations of toxic compounds. A strong inhibition indicates high toxicity of the molecules and more severe negative impact on the bioconversion process (Delgenes et al., 1996). The inhibitory effects have become one of the main bottlenecks to the commercial production of xylitol from LCMs (Canilha et al., 2004, 2008; Marton et al., 2006 and Villarreal et al., 2006). All the inhibitory components need to be removed, or their concentrations reduced so that the hemicellulosic hydrolysate can be effectively used in bioconversion processes (Marton et al., 2006).

2.5.2 Autohydrolysis

Autohydrolysis is an alternative technology for the solubilization of hemicellulose, with various advantages over the dilute acid hydrolysis, namely a more limited delignification and reduced quantities of sugar degradation products (furfural and HMF) (Carvalheiro et al., 2005 and Garrote et al., 2002). Furthermore, it shows some environmental and technical advantages as no chemical (acid or alkali) other than water is employed (Carvalheiro et al., 2005 and El Hage et al., 2010). Autohydrolysis is typically conducted at temperatures of 160–260 ºC, which produces a high-molar mass of xylooligosaccharides without altering substantially the structure of cellulose and lignin, allowing improved recovery during further processing (including enzyme hydrolysis of cellulose or chemical delignification) (Garrote et al., 2001 and Sun and Cheng, 2002). The variables that affect the autohydrolysis efficiency and the hydrolysate composition are residence time, temperature, liquid to solid ratio, and structural integrity of raw material used (Prakasham et al., 2009 and Sun and Cheng, 2002). The mechanism of autohydrolysis reaction is similar to that of dilute acid hydrolysis. This reaction is catalyzed by hydronium ions (H_3O^+). In autohydrolysis, water is the only reactive agent added to substrate, and the reaction includes two stages. In the first stage, hydronium ions coming from water autoionization lead to depolymerization of hemicellulose by selective hydrolysis of both glycosidic linkages and acetyl groups. In the second stage, H_3O^+ coming from acetic acid also acts as a catalyst, improving reaction kinetics. The contribution of H_3O^+ from acetic acid is higher than that from water. During autohydrolysis, acids released from hydrolysis of uronic and acetyl groups present in hemicelluloses catalyze the hydrolysis of bonds

between hemicellulose and lignin as well as that of carbohydrates. Although uronic acids are resistant to hydrolysis, they may also contribute to the formation of H_3O^+ although their role in hydrolysis is still not completely understood (Carvalheiro et al., 2008).

Garrote et al. (2002) studied the production of xylooligosaccharides (XOS) from corncob by autohydrolysis and found that most of the cellulose was retained in the solid residue, whereas partial delignification (up to 26% lignin removal) was achieved. The authors reported that up to 94% of xylan was removed, producing xylooligomers and other byproducts (mainly xylose and furfural). Production of XOS from brewery's spent grain through autohydrolysis has been reported by Carvalheiro et al. (2004b) with a maximum yield of 61% of the feedstock xylan obtained at 190 ºC after 5 min of reaction. It was estimated that 63–77% of the initial xylan was selectively solubilized in autohydrolysis treatments. Nabarlatz et al. (2007) examined six agricultural residues as raw materials for XOS production by autohydrolysis and found that the XOS yield followed xylan content and its accessibility, and further progressively related to the content of acetyl groups in the feedstocks. Analyses of the hydrolysates revealed that they contained partially acetylated oligomeric and polymeric xylan fragments, and a small amount of monosaccharides (e.g., xylose, glucose and arabinose) and degradation products (mainly furfural, HMF and acid soluble lignin). However, the liquor of autohydrolysis is only partly fermentable by microorganisms since the sugars are mainly in the oligomeric form and thus a posthydrolysis step (with dilute acid) is needed to produce the corresponding monosaccharides. A two-step process (autohydrolysis followed by posthydrolysis) is applied to obtain a fermentable hydrolysate (Carvalheiro et al., 2005). Furthermore, autohydrolysis is nonspecific, and other reactions than hemicellulose depolymerization take place, leading to liquor with a complex composition (Garrote et al., 2004). Therefore, autohydrolysis is not feasible for the production of xylose to a satisfactory level.

2.5.3 Enzymatic Hydrolysis

Hydrolysis of LCM by enzyme technology has been justified as an alternative hydrolysis approach. In enzymatic hydrolysis, the utility cost is low compared to

chemical hydrolysis because enzyme hydrolysis is usually performed at mild conditions (pH ~5 and temperature 45–50 ºC) and does not have a corrosion problem (Sun and Cheng, 2002). Compared to acid hydrolysis, enzymatic hydrolysis is milder, environmental friendly, and more specific, but it requires pretreatment to enhance the enzymatic digestibility (Sun and Cheng, 2005). The yield and rate of enzyme-based hydrolysis of LCM is dependent on various parameters such as catalytic properties of enzymes, their loading concentrations, incubation time, raw material type, process variables, pretreatment process, and compounds released during pretreatment method (Zhu et al., 2008). The pretreatment method removes lignin, reduces cellulose crystallinity, and increases the porosity of the materials (Sun and Cheng, 2005). An increase in surface area of cellulose/hemicellulose and enzymes loading improves the hydrolysis rate and time. Among all components in biomass, lignin is considered as a principal barrier to enzyme attack on cellulose, implying the significance of decreasing the structural integrity caused by lignin prior to hydrolysis (Prakasham et al., 2009).

Xylanases and cellulase are the key enzymes used in most of the enzyme treatment studies (Zhu et al., 2008). The digestibility of LCM by enzymes is controlled by the surface area of the raw material, and the increase of surface area through pretreatment or reducing particle size increases the hydrolysis of materials. The application of xylanases alone might not be sufficient due to the complex structure of LCM. Xylanases act synergistically and vary with microbial origin. They cleave the β-(1,4) glycosidic bonds in the xylan molecule releasing short xylooligomers. The choice of a xylanase blend, consisting of xylosidases, arabinofuranosidases, mannanases, hemicellulolytic esterases, and glucuronidases, is one of the significant parameters for effective release of xylose from hemicellulosic material. The choice is also related to the nature of xylan structure, which differs with biomass type (hardwood, softwood, agricultural residue, and grass). Pretreatment either by mild chemical agents at high temperature or by other specific enzyme would present the better hydrolysis method for the efficient recovery of xylose. Though enzyme-based hydrolysis results in higher productivity and/or yield in biotransformation of sugar from pretreated LCM, the price of enzymes is a crucial point in product costing. The use of a hemicellulolytic enzyme mixture is another choice. However, for each material, reaction conditions have to be identified and optimized for the specific enzyme blend. A pretreatment by wet oxidation

is proved to be efficient for LCMs, as a reduction in crystallinity was found along with the degradation of lignin to carboxylic acids, CO_2, and H_2O (Prakasham et al., 2009).

Even with a number of advantages, the major drawback of enzymatic hydrolysis is that the presence of solid residuals (mainly lignin) and dissolution of enzymes in the hydrolysate makes it difficult to separate and recycle the enzymes in order to reduce the cost (Kinnarinen et al., 2012 and Taherzadeh and Karimi, 2007). In addition, enzymes can not freely penetrate the lignocellulosic matrix without pretreatment because of the lignin and thus, the rate of enzyme hydrolysis is slower than acid hydrolysis (Lenihan et al., 2010). Therefore, the feasibility and applicability of enzymatic hydrolysis of hemicellulose requires further study.

2.6 HYDROLYSATE DETOXIFICATION METHODS

The major and common problem associated with efficient bioconversion of xylose to xylitol is that the hemicellulosic hydrolysate contains a broad range of toxic compounds that are inhibitory to xylose-fermenting microorganisms (Villarreal et al., 2006). Four distinct approaches have been reported by Taherzadeh et al. (2000) to minimize the presence of inhibitory components in lignocellulosic hydrolysates: (1) to use bioconversion friendly hydrolysis methods in order to avoid formation of inhibitors; (2) to detoxify the hydrolysate before use for conversion; (3) to construct and/or use inhibitor resistant microorganisms; and (4) to transform toxic compound into nontoxic product that does not interfere with microbial metabolism.

When compared with the microbial fermentation of detoxified hydrolysate or commercial sugar, the fermentation of non-detoxified hydrolysate is characterized by slow kinetics, very limited productivity and yield. Hence, the lignocellulosic hydrolysate needs to be treated and neutralized to achieve the fermentation pH, thereby turning it to be more suitable for microbial assimilation. The selection of the best hydrolysate detoxification approach is significant to improve the efficiency of bioconversion processes. A variety of detoxification methods including physical, chemical and biological treatments have been developed to reduce the concentration of inhibitors or to convert them into inactive compounds (Mussatto and Roberto, 2004).

The effectiveness of a detoxification process depends both on the composition of hemicellulosic hydrolysate and on the species of microbes used because each type of hydrolysate has a different degree of toxicity, and each species of strain has a different degree of tolerance to inhibitors (Larsson et al., 1999). Before selecting a detoxification method, it is important to consider the hydrolysate composition, which varies with the raw materials and the operating conditions applied for hydrolysis (Mussatto and Roberto, 2004). Several methods usually used to remove the toxic compounds and to increase the hydrolysate fermentability, are discussed in the following subsections.

2.6.1 Physical Method

Concentrating hydrolysate through vacuum evaporation is a physical detoxification method to reduce the amounts of volatile compounds such as acetic acid, vanillin, furfural, and HMF found in the hydrolysate (Mussatto and Roberto, 2004 and Prakasham et al., 2009). For the fermentative production of xylitol, Parajó et al. (1997) utilized this approach with wood hydrolysate and noticed an improvement in the concentration of extractives and lignin derivatives. The hydrolysate volume was decreased to about 1/3 (final to initial volume ratio) and the fermentation time necessary for the yeast to consume about 90% of xylose increased from 24 to 94 h. In another study, Silva and Roberto (1999) used vacuum-evaporated rice straw hydrolysate as a substrate for the microbiological production of xylitol from xylose (90 g/L). They observed that the bioconversion was severely hindered by the enhancement in concentration of non-volatile toxic compounds that strongly interfere with fermentation.

Total elimination of furfural from wood hemicellulosic hydrolysate by decreasing its volume by 90% through vacuum evaporation was reported (Larsson et al., 1999). On the other hand, HMF concentration decreased only by 4%. Evaporation is suitable for the removal of acetic acid, furfural and other volatile components from hydrolysate in order to improve the microbial fermentation for xylitol bioproduction (Converti et al., 2000). Rodrigues et al. (2001) utilized the vacuum evaporation either before or after pretreating sugarcane bagasse hydrolysate with activated charcoal. They reported that 98% of furfural was eliminated whereas acetic acid was only partly removed because this compound is volatile in its undissociated form. Recently, Lenihan

et al. (2010) reported that hydrolysate concentration by evaporation is applicable to increase the sugar concentration and to remove microbial growth inhibitors such as acetic acid, furfural and HMF. Vacuum evaporation is the most promising physical detoxification process for enhancing the biotechnological production of xylitol (Parajó et al., 1996). However, this process enhances the concentration of non-volatile inhibitory compounds (extractives and lignin derivatives) and consequently elevates the degree of fermentation inhibition (Mussatto and Roberto, 2004). The physical method is also ineffective to eliminate phenolic compounds from the hydrolysate. Therefore, it is important to find an efficient method that can eliminate and/or reduce all inhibitory compounds from hemicellulosic hydrolysates for a better bioconversion process.

2.6.2 Chemical Method

For reducing the toxicity of the lignocellulosic hydrolysate, some chemical methods capable of inducing precipitation of the toxic compounds include: pH adjustment (Martinez et al., 2001), detoxification with ion-exchange resins (IERs) (Carvalho et al., 2004), adsorption on activated charcoal (Mussatto and Roberto, 2001) or on diatomaceous earth (Ribeiro et al., 2001). These methods can be employed separately, but lately there has been a tendency to use them in different combinations (Canilha et al., 2004).

Adjustment of hydrolysate pH is an effective and the cheapest chemical treatment among available methods. In general, $Ca(OH)_2$ and H_2SO_4 are employed for the treatment of hydrolysate to remove phenolic compounds, ketones, furfurals and HMF (Nilvebrant et al., 2001 and Rao et al., 2006). Hydrolysate detoxification using alkali treatment by improving the pH to 9.0–10.0 with $Ca(OH)_2$ (overliming) and then readjusting the pH to 5.5 with H_2SO_4 has been reported as early as 1945 by Leonard and Hajny. The detoxifying effect of overliming is due both to the precipitation of toxic compounds and to the instability of some inhibitors at high pH (Palmqvist and Hanh-Hägerdal, 2000a). Zyl et al. (1988) reported that the use of $Ca(OH)_2$ to adjust the pH results in better fermentability than using NaOH due to the precipitation of toxic compounds. Palmqvist and Hanh-Hägerdal (2000a) utilized NaOH and $Ca(OH)_2$ to adjust the pH to 10.0 of dilute acid hydrolysate of spruce and obtained a 20% reduction

of furfural and HMF but the concentration of acetic acid remained unchanged. Martinez et al. (2001) reported that the use of $Ca(OH)_2$ to adjust the pH of sugarcane bagasse hemicellulosic hydrolysate (overliming) to 9.0 proved to be a very efficient detoxification method. Mohagheghi et al. (2006) reported that employing $Ca(OH)_2$ to adjust the pH of corn stover hydrolysate to 11.0 was highly fermentable, but xylose losses were highest (up to 34%) at this condition.

Treatment with activated charcoal is another technique drawing much attention due to its low cost and a high capacity to absorb pigments, free fatty acids, n-hexane and other oxidation products (Rao et al., 2006). Activated charcoal is also capable of adsorbing toxic components such as phenolics, acetic acid, aromatic compounds, furfural and HMF normally found in hemicellulosic hydrolysate (Canilha et al., 2004; Dominguez et al., 1996; Mussatto and Roberto, 2001 and Silva et al., 1998). Dominguez et al. (1996) also verified several types of treatments for sugarcane bagasse hydrolysate (neutralization, treatment with charcoal and IERs). Most of the inhibiting substances were eliminated, and xylitol production rate was the highest (0.205 g/L·h) when the hydrolysate was treated with activated charcoal. According to Mussatto and Roberto (2001), a detoxification method using activated charcoal with concentrated rice straw hydrolysate improved the conversion of xylose to xylitol with *Candida guilliermondii* by 22%. When the hydrolysate was treated with charcoal (hydrolysate:charcoal ratio 40 g/g), 27% of phenolic compounds was removed, the xylitol yield and productivity reaching 0.72 g/g and 0.61 g/L·h, respectively. The effectiveness of charcoal treatment depends on several process parameters such as solid to liquid ratio, contact time, temperature and pH (Prakasham et al., 2009).

Adsorption on IERs is also an effective technique, but its cost is high compared to that of other treatments (Lee et al., 1999). To detoxify sugarcane bagasse hydrolysate and to improve xylitol production by calcium alginate-entrapped yeast cells, Carvalho et al. (2004) described the performance of four different IERs in sequence: A-103S, A-860S, Applexion cation, and anion. Anion-exchange resins were more efficient for removing phenols and furans than cation-exchange resins. This treatment resulted in a removal of 82.1% furfural, 66.5% HMF, 61.9% phenolic compounds, 100% chromium, 28.5% iron, 46.1% zinc, 14.7% sodium and 3.5% nickel. On the other hand, the

elimination of acetic acid was not significant by this treatment. A comparative evaluation of various chemical detoxification methods indicated that anion exchange resins remove high percentages of toxic compounds such as acetic acid (96%), phenolic compounds (91%), furfural (73%), HMF (70%), in addition to substantial removal of, aldehydes and aliphatic acids from hydrolysate compared to cation exchange resins (Mussatto and Roberto, 2004 and Rao et al., 2006).

2.6.3 Biological Method

Biological method of detoxification involves the utilization of specific enzymes or microbes that serve on the inhibitory components present in the hydrolysates and alter their composition (Mussatto and Roberto, 2004). Treatment of willow wood hydrolysate with laccase and peroxidase enzymes from the ligninolytic fungus *Trametes versicolor*, has been shown to increase the ethanol productivity 2–3 times (Jönsson et al., 1998). The laccase treatment led to selective and virtually complete removal of phenolic monomers and acids. The mechanism of detoxification of these enzymes possibly includes oxidative polymerization of low molecular-mass phenolic compounds (Mussatto and Roberto, 2004).

The adaptation of microbial cells to the hydrolysate containing inhibitors is an attractive biological approach for enhanching the fermentability of hemicellulosic hydrolysate media (Nigam, 2001; Parajó et al., 1998b; Rao et al., 2006; Sene et al., 2001; Silva and Roberto, 2001b and Zeid et al., 2008). This technique is based on successive fermentations, which utilize the microorganism from one experiment as the inoculum for the next (Silva and Roberto, 2001b). Silva and Roberto (2001b) studied on the synthesis of xylitol from rice straw hemicellulosic hydrolysate by adapted and un-adapted strain of *Candida guilliermondii*. The yield of xylitol increased about 3-fold (from 17 to 50 g/L), and xylose consumption increased from 52 to 83%, after 120 h of fermentation using adapted yeast in the hydrolysate containing 90 g/L xylose. The authors concluded that adaptation of yeast cells to hydrolysate is an inexpensive and effective technique to alleviate the inhibitory effects of toxic components on the conversion of xylose to xylitol. Mussatto and Roberto (2003) also performed batch fermentation with rice straw hydrolysate containing about 85 g/L xylose in a bioreactor

at 30 ºC for the bioconversion of xylose to xylitol by the adapted yeast *C. guilliermondii*. The highest xylitol yield (0.84 g/g) was achieved without pretreating the hydrolysate. In order to improve yeast growth and xylitol production from hydrolysate containing inhibitors, Rao et al. (2006) prepared adapted cell of *C. tropicalis* by subculturing in the hydrolysate containing medium for 25 cycles. It was revealed that this organism produced more xylitol (0.58 and 0.65 g/g of xylose) than the parent strain with corn fiber and sugarcane hydrolysates, respectively. Inoculating the rice straw hydrolysate medium with the adapted yeast cells of *C. guilliermondii* and *C. tropicalis* produced the maximum yield (47.35 and 45.2 g/L xylitol out of 60 g/L xylose i.e., 0.79 and 0.75 g/g) after 96 h of incubation for both strains, respectively. The highest yield of xylitol indicated the efficacy of adapted cells for the bioconversion of xylose to xylitol (Zeid et al., 2008). It can be concluded that cell adaptation is a low cost technique which helps the bioconversion of LCM hydrolysate and provides an alternative to the detoxification methodologies (Silva and Roberto, 2001b). However, one of the critical issues concerned with biological methods is their long time requirement. Hence, it warrants for further investigation of an effective and economical approach for the detoxification of the LCM hydrolysate. When detoxification enhances the production costs, it is necessary either to bypass the detoxification steps or to develop efficient and cheap methods (Carvalheiro et al., 2005). The construction of a genetically engineered new microbial strain that tolerates inhibitors would be a better option because it would remove detoxification steps.

2.6.4 Combined Treatment

The best outcomes can only be achieved by combining two or more distinct detoxification methods because each method is specific to certain types of toxic components (Mussatto and Roberto, 2004). Converti et al. (1999) observed that the presence of both acetic acid and lignin-derived compounds hindered the fermentative production of xylitol from hardwood hydrolysate by yeast strains. To detoxify the hydrolysate and improve the bioconversion kinetics, they applied a combination of three treatments: overliming, charcoal adsorption, and evaporation. The combination of these treatments allowed efficient transformation of xylose to xylitol by *Pachysolen tannophilus*. After 96 h of fermentation, 39.5 g/L xylitol was obtained from 89 g/L

xylose, which corresponds to a volumetric productivity of 0.41 g/L·h and a yield of 0.63 g/g in relation to the substrate consumed. Carvalho et al. (2006) compared different procedures for the detoxification of eucalyptus hydrolysate for use in fermentative processes including concentration by vacuum evaporation and adsorption on activated charcoal, ion-exchange resin or adsorbent resin, diatomaceous earths. Vacuum evaporation was especially effective to remove furfural, whereas the adsorbent resin was efficient in removing HMF, acetic acid and phenolics. The combination of adsorbent resin with charcoal was better than with diatomaceous earths for the removal of acetic acid and phenolic compounds. The best detoxification method evaluated was based on hydrolysate concentration followed by adsorption on charcoal and adsorbent resin. By this combined treatment, removal rates of 82.5, 100, 100 and 94% were obtained for acetic acid, furfural, HMF and phenolics, respectively. It is important to identify the major inhibitors present in the hydrolysate before choosing a method or a sequence of methods for detoxification. This knowledge not only helps to choose an efficient and low cost detoxification method, but also to set up the hydrolysis conditions that diminish the formation of inhibitors.

2.7 XYLITOL PRODUCTION PROCESSES

Xylitol can be produced by chemical or biological reduction of pure D-xylose or xylose-rich lignocellulosic hydrolysate. Birch wood, oats, cotton-seed hulls, corn fiber, corncobs, sugarcane bagasse, rice straw, nut shells and brewer's spent grain are xylan-rich materials, which could be utilized for the synthesis of xylitol (Affleck, 2000 and Mussatto and Roberto, 2005). The comparatively high added-value and growing market for xylitol have fostered extensive research on its production. Figure 2.4 summarizes the different methods of xylitol production.

2.7.1 Chemical Process

The chemical process for xylitol manufacture was developed in the 1970s in Finland (Mäkinen, 2000a). This process is divided into two groups namely solid-liquid extraction and catalytic reduction, which are discussed in the following subsections.

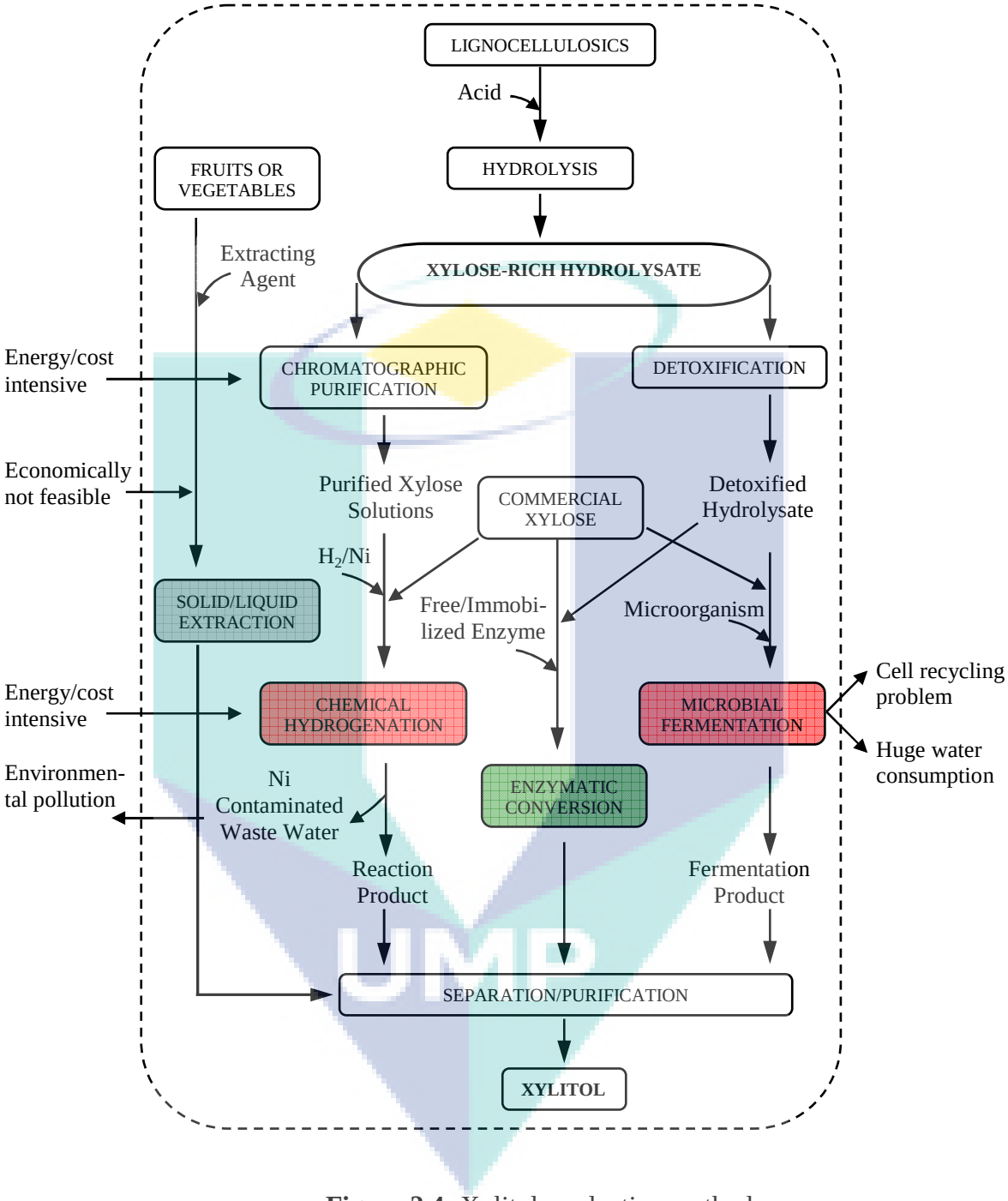


Figure 2.4: Xylitol production methods

Source: adapted from Parajó et al. (1998a)

Solid-Liquid Extraction

Xylitol is naturally found in small amounts in many fruits and vegetables (banana, grape, yellow plum, strawberry, raspberry, lettuce, carrot, cauliflower, onion, etc.), as well as in yeasts, lichens, seaweeds and mushrooms. It can be extracted from these sources through solid/liquid extraction, but such process would not be economically feasible due to the relatively low xylitol content and high cost of the raw materials (Chen et al., 2010; Parajó et al., 1998a and Winkelhausen and Kuzmanova, 1998). Therefore, extensive research has been focused on xylitol production by chemical or biotechnological processes.

Chemical Reduction

Xylitol is industrially manufactured by reducing pure xylose achieved from hardwood hemicellulosic hydrolysate in the presence of a Raney nickel catalyst (Granström et al., 2007a; Melaja and Hämäläinen, 1977 and Ojamo et al., 2009). The chemical process for manufacturing xylitol consists of four major steps (Figure 2.4): (i) hydrolysis of lignocellulosic biomass by mineral acid, (ii) purification and separation of the hydrolysate to obtain pure xylose as solution or in crystalline form, (iii) catalytic reduction of the xylose to xylitol, and (iv) crystallization and separation of the xylitol. The chemical synthesis of xylitol begins with the recovery of xylose from hemicellulosic material by acid-catalyzed hydrolysis. The amorphous and noncrystalline structure of hemicellulose allows easy diffusion of the hydronium ions in the polymer matrix, favoring the hydrolysis reaction. The hydrolysates generally contain a variety of sugars (xylose, arabinose, glucose, galactose, and mannose) in proportions that are dependent on the biomass type and experimental conditions. After purification and color removal, xylose-containing hemicellulosic hydrolysate can be used for manufacturing xylitol by reduction of xylose at 80–140 °C and hydrogen pressures up to 50 atmospheres in the presence of metal catalysts (Raney nickel). The xylitol solution formed by catalytic reduction requires further chromatographic purification, concentration and crystallization of the product to achieve pure xylitol (Melaja and Hämäläinen, 1977). Xylitol yield is only about 50–60% of the xylan fraction or 8–15% of the starting raw material. Therefore, the xylitol production process is expensive due

to the extensive separation and purification steps (Kamal et al., 2011; Nigam and Singh, 1995; Parajó et al., 1998a and Saha, 2003). Chemical methods are technically complicated multi-step processes that have relatively low efficiency. The greatest problem lies in achieving a complete and effective separation of xylose from other hydrolysis byproducts. Thorough purification is essential because the catalysts employed in the hydrogenation of xylose are very sensitive to byproducts (Ojamo et al., 2009). Despite a large number of drawbacks, the advantages of the chemical process are: (i) it provides a xylose solution of sufficiently high purity that ensures specific hydrogenation to yield xylitol on a commercial-scale, (ii) nonhydrogenated sugars are easily separated from the mixture of polyols by ion exchange chromatographic techniques, and (iii) provides easy separation of desired polyols from the mixture after hydrogenation of the wood hydrolysates (containing 75% (w/w) xylose and 25% other sugars (such as glucose, mannose, galactose, and arabinose)) (Melaja and Hämäläinen, 1977). The processes discussed in this chapter for xylitol production with their yields, productivities, advantages, and disadvantages are summarized in Table 2.4.

2.7.2 Biotechnological Processes

Due to the use of elevated temperature and pressure as well as the need for expensive separation and purification steps in the chemical reduction process, xylitol manufacture through bioconversion has been proposed as an alternative process (Liaw et. al., 2008). Biotechnological processes for the production of xylitol are based on the use of microorganisms or isolated enzymes. In view of alternatives to the chemical process, two biotechnological processes seem promising: the microbial process and the enzymatic approach employing isolated XR.

Microbial Process

For more than last four decades, xylitol production by microbial fermentation has been thoroughly studied as an alternative to the chemical process. The microbiological process uses bacteria, fungi, yeast, and recombinant strains to produce xylitol from pure xylose or a hemicellulosic hydrolysate. Production of xylitol using microorganisms is summarized below.

Table 2.4: Xylitol production processes with their yields, productivities, advantages, and disadvantages

Method	Raw material	Hydrogenation agent	$Y_{p/s}$ [a] (%)	Q_p [b] (g/L·h)	Advantage	Disadvantage	Reference
Chemical	Xylan-containing materials	H_2/Ni	50–60	n.a.	It provides a highly purified xylose that ensures hydrogenation Nonhydrogenated sugars are easily separated from the mixture of polyols	Laborious and cost/ energy-intensive Use of extensive separation and purification steps Requirement of high temperature and pressure Low efficiency process Not environmental friendly	(Melaja and Hämäläinen, 1977; Nigam and Singh, 1995 and Parajó et al., 1998a)
Microbial	D-xylose	Wild-type yeast	65–85	2.67–12	Cost effective due to the nonnecessity of xylose purification and low energy consumption High productivity and moderate yield Environmental friendly	Time-consuming Cell recycling problem Huge water consumption Downstream processing problem regarding the media ingredients	(Kwon et al., 2006; Parajó et al., 1998a and Prakasham et al., 2009)
		Recombinant yeast	86–100	1–2.34			(Bae et al., 2004 and Govinden et al., 2001)
Enzymatic	D-xylose	XR from yeast	96	2.8–3.33	No cell recycling limitation Eco-environmentally more attractive than microbial process as it has the potential for energy and water savings High yield and productivity Easy recovery of xylitol	Cost of XR preparation Coenzyme regeneration	(Neuhauser et al., 1998 and Nidetzky et al., 1996)

[a] $Y_{p/s}$: xylitol yield on xylose consumed (%); [b] Q_p: xylitol volumetric productivity (g/L·h); n.a.: not available

Bacterial Production of Xylitol

It has been reported that a few bacteria, such as *Enterobacter liquefaciens*, *Corynebacterium* sp., *Mycobacterium smegmatis*, and *Gluconobacter oxydans*, produce xylitol in small amounts. Screening for xylose-utilizing bacteria by Yoshitake et al. (1973) showed that an *Enterobacter* strain cultured on D-xylose and produced xylitol extracellularly. The production of xylitol by the *Enterobacter* strain using D-xylose was by NADPH-dependent XR; this proved that enzymatic bioconversion was not confined to fungi and yeasts. This strain was reported to yield 33.3 g/L xylitol in a cultivation medium containing 100 g/L initial xylose for 4 days with a productivity of 0.35 g/L·h. The *Corynebacterium* species produced xylitol only when grown in media having both D-xylose and gluconate. In another experiment, seventeen cultures belonging to three genera of facultative bacteria were screened by Rangaswamy and Agblevor (2002), and *Corynebacterium* sp. B-4247 produced the highest amount of xylitol among the screened cultures. The maximum xylitol yield produced in 24 h was 0.57 g/g xylose using an initial xylose concentration of 75 g/L. About 80% xylitol production yield was reported by Izumori and Tuzaki (1988) with D-xylose as the substrate using immobilized D-xylose isomerase (XI; EC 5.3.1.5) and *Mycobacterium smegmatis*. Suzuki et al. (2002) tested 420 bacterial strains based on their xylitol-producing capacity from D-arabinitol and observed that *Gluconobacter oxydans* was the best xylitol producer among the isolates, with a yield of 29.2 g/L xylitol from 52.4 g/L D-arabinitol after 27 h incubation using intact cells as the enzyme source. However, xylose-fermenting bacteria do not currently attract researchers' interest due to the relatively low amount of xylitol produced.

Fungal Production of Xylitol

The production of xylitol using fungi has been studied to a lesser extent. In xylitol production experiments, the filamentous fungi *Aspergillus, Byssochlamys, Gliocladium, Myrothecium, Penicillium, Rhizopus,* and *Neurospora* sp. have been shown to produce small quantities of xylitol in xylose-containing media (Chiang and Knight, 1960). Ueng and Gong (1982) detected low amounts of xylitol in the fermentation of *Mucor* sp. on sugarcane bagasse hemicellulose hydrolysate. Suihko

(1984) reported 1 g/L of xylitol production by *Fusarium oxysporum* when grown 2 days in a medium containing 50 g/L of initial D-xylose under aerobic conditions. There is only one significant report regarding the capacity of the fungi *Petromyces albertensis* to produce xylitol. Dahiya (1991) studied xylitol production using *P. albertensis* and detected a yield of 0.4 g/g xylose after 10 days of incubation in a fermentation medium containing 100 g/L D-xylose.

Production of Xylitol by Yeast

Xylitol is produced as a metabolic intermediate compound in all organisms whose xylose metabolism takes place in a sequential activity of XR and xylitol dehydrogenase (XDH; EC 1.1.1.9) enzymes. Keeping this in view, various researchers have been involved in microbial screening studies to identify efficient strains for the production of xylitol. Several wild-type and recombinant yeasts have been used for the fermentative production of xylitol (Table 2.4). Biological production of xylitol by yeasts has been reported (Onishi and Suzuki, 1969), especially by *Candida* sp. such as *Candida pelliculosa*, *C. boidinii*, *C. guilliermondii*, and *C. tropicalis* and *Pachysolen tannophilus*. Fifteen yeast strains were compared for xylose bioconversion (Gong et al., 1981), with a mutant of *Candida tropicalis* HPX2 giving the highest xylitol yield of above 0.90 g/g from 20% D-xylose. In another experiment, Gong et al. (1983) tested 20 strains of eleven species of *Candida*, 21 strains of eight species of *Saccharomyces*, and 8 strains of *Schizosaccharomyces pombe*. All of the *Candida* sp. produced xylitol (10–15%, w/v). The XDH gene (*XYL2*)-disrupted mutant of *C. tropicalis* synthesized xylitol from D-xylose using glycerol as a cosubstrate with the xylitol yield and volumetric productivity of 97% (w/w) of the theoretical yield and 3.2 g/L·h, respectively (Ko et al., 2006).

Forty-four yeast strains from the five genera of *Candida, Kluyveromyces, Hansenula*, *Pachysolen*, and *Pichia* were screened by Barbosa et al. (1988) for their ability to convert D-xylose to xylitol. *Candida guilliermondii* and *C. tropicalis* were found to be the highest xylitol producers. These yeasts produced 77.2 g/L xylitol from 104 g/L D-xylose using high cell densities and a defined medium under aerobic conditions. A volumetric productivity of 2.67 g/L·h xylitol with 172 g/L initial D-xylose

as substrate was obtained by Horitsu et al. (1992) using *C. tropicalis*. The fermentation conditions were optimized by da Silva and Afschar (1994) during continuous cultivation of *Candida tropicalis* DSM 7524 for xylitol production. *C. tropicalis* produced xylitol at a yield of 77–80% of the theoretical value (0.91 g/g) in a medium containing 100 g/L D-xylose. Among the tested yeasts, Vandeska et al. (1995) selected *Candida boidinii*, which gave a higher xylitol yield (0.47 g/g), corresponding to 52% of the theoretical yield, with 150 g/L xylose after 14 days. *Candida mogii* gave the highest yield of 0.62 g/g in comparison with eleven other D-xylose-utilizing yeasts studied by Sirisansaneeyakul et al. (1995). In xylitol production experiments, Dominguez et al. (1996) compared six known xylitol-producing yeast strains and concluded that *Debaryomyces hansenii* was an efficient xylitol producer, with a yield of 0.71 g/g. Ikeuchi et al. (1999) screened xylitol-producing microorganisms from soils using xylose as sole carbon source and selected strains classified as *Candida* sp. according to a taxonomic identification. They reported that *Candida* sp. 559-9 was a potential candidate for xylitol production, with a yield of 173 g/L from 200 g/L xylose after 5 days of incubation. In another investigation, Suryadi et al. (2000) tested four methanol-assimilating yeasts for xylitol synthesis from xylose. *Hansenula polymorpha* was identified as a good xylitol producer out of four strains screened, with 58 g/L xylitol production from 125 g/L xylose after 4 days of incubation. Kim et al. (2004) produced xylitol from xylose through the long-term cell-recycle fermentation of *C. tropicalis* using a chemically defined medium that achieved a volumetric productivity of 5.4 g/L·h and a yield of 81% (w/w).

To increase production rate and to prevent the loss of xylitol-producing microbial biocatalysts in a fed-batch submerged membrane bioreactor, Kwon et al. (2006) employed a hollow-fiber membrane. The authors observed that *C. tropicalis* produced xylitol at a yield and production rate of 85% (w/w) and 12 g/L·h, respectively, from an initial xylose concentration of 200 g/L with glucose as cosubstrate (20 g/L). This is the highest volumetric productivity among the reported values in the literature. Guo et al. (2006) selected 5 strains for further study after screening 274 yeasts for xylitol production and identified *Candida maltosa* and *C. guilliermondii* as the best xylitol producers. A total of 35 yeast strains isolated from beetles gut showed a variation in their ability to synthesize xylitol from xylose (Rao et al., 2007). Among

them *Pichia* sp. produced highest amount of xylitol (0.58 g/g xylitol). Recently, 270 yeast isolates were screened by Sampaio et al. (2008). The authors concluded that *Debaryomyces hansenii* UFV-170 was the best xylitol producer among the tested isolates, producing 5.84 g/L xylitol from 10 g/L xylose after 24 h cultivation, corresponding to a yield of 0.54 g/g xylose.

More recently, Huang et al. (2011) developed a yeast strain, *Candida tropicalis* JH030 for xylitol production from hemicellulosic hydrolysate without detoxification. The newly isolated strain gave a promising xylitol yield of 0.71 g/g from nondetoxified rice straw hydrolysate that had been made by the dilute acid pretreatment under severe conditions. The nonnecessity of hydrolysate detoxification indicates the potential of this yeast for xylitol production. A xylitol assimilation-deficient mutant strain, *C. tropicalis* SS2, was created via chemical mutagenesis for xylitol production in batch and fed-batch cultures (Jeon et al., 2011). Batch fermentation with this mutant produced a six-fold higher xylitol yield than the parent strain in a medium containing 25 g/L glucose and 25 g/L xylose. The mutant strain showed the potential for xylitol production by fed-batch fermentation with repeated addition of glycerol (as an alternative low cost nonfermentable carbon source) and xylose that resulted in 3·3 g/L·h xylitol productivity and overall xylitol yield of 0·93 g/g in a final concentration of 220 g/L under aerobic condition. It is confirmed from the screening results of different xylitol-producing microbes that yeasts, in general, and *Candida* genus are particularly involved in xylitol synthesis (Nigam and Singh, 1995; Winkelhausen and Kuzmanova, 1998 and Zhang et al., 2012). However, the costs and times associated with hydrolysate detoxification for fermentative production of xylitol helps to perceive that the development and utilization of inhibitor tolerant organisms offers a more acceptable biotechnological method to synthesize this compound from crude xylose feedstocks. Table 2.5 summarizes the reported data on the fermentative production of xylitol by different microbial strains using pure xylose or lignocellulosic hydrolysate. Due to the difference in physiological nature of microbial strains and variation in composition of hydrolysates used, comparison of their fermentation efficiency is not so easy. Still, the table will give the reader a sense of the performance of each strain.

Table 2.5: Reported data on the microbial production of xylitol from pure xylose or from lignocellulosic hydrolysate

Microorganisms	Fermentation conditions	Raw material	Time (h)	P [c] (g/L)	$Y_{p/s}$ [a] (g/g)	Q_p [b] (g/L·h)	Reference
Candida tropicalis HPX2	30 ºC, v/V 100/250 mL, X_0 20 g/L, 200 rpm	D-xylose	24	40	>0.90	1.67	Gong et al., 1981
C. tropicalis BSXDH-3	30 ºC, v/V 1/2.5 L, X_0 50 g/L, 300-500 rpm	D-xylose Glycerol (20 g/L)	n.a.	n.a.	0.97	3.2	Ko et al., 2006
C. guilliermondi FTI-20037	30 ºC, v/V 100/250 mL, X_0 104 g/L, 200 rpm	D-xylose	78	77.2	0.74	0.99	Barbosa et al., 1988
C. tropicalis IFO 0618	30 ºC, v/V 200/250 mL, X_0 172 g/L	D-xylose	n.a.	n.a.	0.64	2.67	Horitsu et al., 1992
C. tropicalis DSM 7524	30 ºC, X_0 100 g/L, 300 rpm, pH 2.5	D-xylose	800	220	0.70–0.73	0.28	da Silva and Afschar, 1994
C. boidinii NRRL Y-17213	30 ºC, v/V 50/125 mL, X_0 150 g/L, 125 rpm	D-xylose	336	53.1	0.47	0.16	Vandeska et al., 1995
C. mogii ATCC 18364	30 ºC, v/V 300/1000 mL, X_0 53 g/L, 250 rpm	D-xylose	n.a.	n.a.	0.62	n.a.	Sirisansaneeyakul et al., 1995
Debaryomyces hansenii NRRL Y-7426	28 ºC, v/V 10/50 mL, X_0 150 g/L, 180 rpm	Sugarcane bagasse	48	10.54	0.71	0.22	Dominguez et al., 1996
Candida sp. 559-9	30 ºC, v/V 10/30 mL, X_0 200 g/L, 110 rpm	D-xylose	120	173	0.90 (ca.)	1.44	Ikeuchi et al., 1999
Hansenula polymorpha	30 ºC, v/V 200/500 mL, X_0 125 g/L, 150 rpm	D-xylose Glycerol (5%)	96	58	0.62	0.60 (ca.)	Suryadi et al., 2000
C. tropicalis	30 ºC, v/V 2/7 L, X_0 150 g/L, 350-400 rpm, 14 recycle rounds	D-xylose	284	110	0.81	5.4	Kim et al., 2004

Table 2.5: Continued

Microorganisms	Fermentation conditions	Raw material	Time (h)	P [c] (g/L)	$Y_{p/s}$ [a] (g/g)	Q_p [b] (g/L·h)	Reference
C. tropicalis KCTC 10457	30 ºC, v/V 2/5 L, X_0 200 g/L, 350 rpm, 10 recycle rounds	D-xylose Glucose (20 g/L)	152	1824 (ca.)	0.85	12	Kwon et al., 2006
Pichia sp.	28 ºC, v/V 100/250 mL, X_0 40 g/L ,250 rpm	D-xylose	50	25	0.58	0.5 (ca.)	Rao et al., 2007
D. hansenii UFV-170	30 ºC, v/V 25/125 mL, X_0 10 g/L, 200 rpm	D-xylose	24	5.84	0.54	0.24	Sampaio et al., 2008
C. tropicalis JH030	30 ºC, v/V 60/250 mL, X_0 46 g/L, 150 rpm	Rice straw	71 (ca.)	31.1	0.71	0.44	Huang et al., 2011
C. tropicalis SS2	X_0 25 g/L	Xylose Glucose (25 g/L)	67 (ca.)	220	0.93	3.3	Jeon et al., 2011

[a] $Y_{p/s}$: xylitol yield on xylose consumed (g/g); [b] Q_p: xylitol volumetric productivity (g/L·h); [c] P: maximum xylitol production (g/L); n.a.: not available; v/V: (volume medium/volume system); X_0: initial xylose concentration (g/L); ca.: calculated

Production of Xylitol by Recombinant Strains

A large number of recombinant *Saccharomyces cerevisiae* strains have been constructed by expressing the xylose reductase gene (*XYL1*) from *Pichia stipitis* and *C. shehate.* The production of xylitol from xylose using these transformants in batch, fed-batch, and cell-recycle fermentations have been studied (Bae et al., 2004; Chung et al., 2002; Govinden et al., 2001; Hallborn et al., 1991; Lee et al., 2000 and Meinander et al., 1994). These strains required a cosubstrate (glucose, ethanol) for the regeneration of reduced coenzymes used in the reduction of xylose and for the maintenance and growth of cells. The recombinant yeasts presented a high xylitol conversion yield ranging from 86–100% of the theoretically expected value (Table 2.4), but the volumetric productivity (1.0–2.34 g/L·h) was lower than that of wild-type xylose-fermenting yeasts such as *C. tropicalis* (5.4 g/L·h) (Kim et al., 2004). Among most of these transformants, the initial xylose concentration did not exceed 20 g/L (Hallborn et al., 1991; Meinander et al., 1994 and Winkelhausen and Kuzmanova, 1998).

Since native *E. coli* does not have the capability to synthesize xylitol, a redesign strategy for that strain was employed involving a foreign metabolic pathway (Akinterinwa et al., 2008 and Cirino et al., 2006). In order to improve the capabilities of bacteria to overproduce sugar alcohols, especially xylitol, a variety of interesting metabolic engineering efforts have been seen in the past few years (Akinterinwa et al., 2008, 2009). To test the expression of XR gene isolated from *Candida boidinii* (CbXR) in *E. coli*, xylitol was produced from a mixture of glucose and xylose, glucose serving as the source of energy and reducing equivalents. Expression of NADPH-dependent CbXR in the mutant strain PC09 (DeltaxylB, crp* mutant) in a batch fermentation containing minimal medium resulted in the production of ~250 mM xylitol (38 g/L in 46 h), with concomitant utilization of ~150 mM glucose (Cirino et al., 2006). A xylitol-producing recombinant *E. coli* strain was constructed by chromosomal insertion of XR from *Kluyveromyces lactis* (*XYL1*) and *E. coli* xylose permease (*xylE*) under the control of an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter (Hibi et al., 2007). The gene disruption study indicated that a *yhbC*-deficient strain showed the highest improvements in xylitol productivity (increasing from 0.68 to 0.81 g/L·h) among the tested mutants. Khankal et al. (2008) reported that by overexpressing the

ATP-dependent XylFGH xylose transporter from *E. coli* resulted in an average specific xylitol productivity of 0.33 g/g·h in a fed-batch fermentation using defined mineral salts medium. The obtained specific productivity is considerably higher than that reported for *C. tropicalis* (0.22 g/g·h) and significant improvements are expected from high density resting cells with optimized oxygen control.

Production of xylitol from hemicellulosic feedstocks by engineered *E. coli* has the potential to become a commercially viable process (Akinterinwa et al., 2008). Nyyssölä et al. (2005) reported that xylitol was produced from D-xylose in a recombinant *Lactococcus lactis* strain by expressing the XR gene from *P. stipitis* (*XYL1*) while metabolizing glucose as the energy source. The glucose-limited fed-batch fermentation with high xylose concentration (160 g/L) produced 1.0 mol xylitol per mol xylose and 2.5 mol xylitol per mol glucose at a volumetric productivity of 2.72 g/L·h for 20 h. The authors also reported that the coexpression of the xylose transporter gene with XR did not improve xylitol production appreciably.

Xylitol production from glucose by metabolically engineered strains of *Bacillus subtilis* was demonstrated (Povelainen and Miasnikov, 2007). The expression of xylitol phosphate dehydrogenase (XPDH) gene isolated from *Clostridium difficile* or *Lactobacillus rhamnosus* in a pentulose-producing mutant of *B. subtilis* (GX7) resulted in xylitol production with a yield of ~23% in LB medium containing 10% glucose. More recently, Cheng et al. (2011) studied xylitol production from xylose mother liquor via a novel strategy that combines the use of recombinant *B. subtilis* and *C. maltosa*. They developed a biological method for the detoxification of xylose mother liquor by *C. maltosa* and the purification of xylose from detoxified mother liquor using *B. subtilis*. Subsequently, xylitol was prepared by *C. maltosa*-mediated biohydrogenation of xylose with 4.25 g/L·h volumetric productivity. Though this has been shown to be a highly productive process, involvement of two microbial processing steps (detoxification and xylitol production) renders it expensive and time-consuming. In fact, the authors (Cheng et al., 2011) did not count the detoxification time while calculating productivity.

Xylose reductases (XRs), in general, show relaxed sugar specificity and are able to reduce L-arabinose, another major component of some hemicellulosic hydrolysates (such as hardwood hydrolysate) (Akinterinwa et al., 2008, and Nair and Zhao, 2008). Promiscuous substrate specificity is problematic when xylitol is the desired product from LCMs containing D-xylose, L-arabinose and other sugar monomers. The XR enzyme was purified and characterized from *Neurospora crassa* (NcXR) and found to have higher selectivity for D-xylose over L-arabinose (2.4-fold), compared to several other XRs (Woodyer et al., 2005). Easy isolation of this enzyme, coupled with its high activity and catalytic efficiency, may prove useful in the *in vitro* production of xylitol. To improve the selectivity toward xylitol production, Nair and Zhao (2008, 2010) engineered the NcXR for reduced L-arabinose reductase activity and, via various rounds of directed evolution, found a mutant NcXR (VMQCI) that had a 50-fold lower catalytic efficiency to L-arabinose. The engineered *E. coli* strain in conjunction with the VMQCI was able to eliminate arabinitol production from an equiweight mixture of xylose, arabinose, and glucose.

Regulatory Factors in Microbial Xylitol Production

The microbial production of xylitol has been studied as an alternative, but its viability is dependent on the optimization of the various fermentation variables (Branco et al., 2009). All research papers reporting the bioconversion of xylose to xylitol using microbial strains have demonstrated that xylitol production is influenced by a number of experimental conditions. Investigating the effects of these conditions is of particular interest as a prerequisite for maximum xylitol yields and productivities. Xylitol production by yeasts is generally influenced by the nutritional composition (substrate, nitrogen source, and micronutrients and their concentrations), the culture and process conditions (temperature, pH, aeration, inoculum concentration, immobilization, and reactor conditions), and the genetic nature of the microorganisms (native isolates, mutants, and recombinant strains) (Branco et al., 2009; da Silva and Afschar, 1994; Horitsu et al., 1992; Nigam and Singh, 1995; Prakasham et al., 2009 and Winkelhausen and Kuzmanova, 1998).

Enzymatic Process

The production of xylitol from xylose using enzyme technology is an alternative and promising approach (Table 2.4). The XR-mediated reduction of D-xylose is NAD(P)H dependent; thus, one mole of coenzyme is consumed per mole of xylitol produced (Nidetzky et al., 1996). The enzymatic conversion of D-xylose into xylitol using the XR of *Candida pelliculosa* coupled with the oxidoreductase system of *Methanobacterium* sp. (for $NADP^+$ to NADPH reduction with H_2 gas as an electron donor) has been reported by Kitpreechavanich et al. (1984). Xylose was stoichiometrically converted to xylitol with an equivalent consumption of NADPH, and an almost quantitative conversion of xylose to xylitol was achieved using a $NADP^+$-to-xylose ratio of more than 1:30; the coenzyme was successfully regenerated and retained using a membrane reactor. About 90% conversion of xylose to xylitol could be achieved at 35 °C and pH 7.5 after a 24 h reaction period. Nidetzky et al. (1996) optimized the production of xylitol from xylose by XR from *Candida tenuis* coupled with glucose dehydrogenase from *Bacillus cereus* for regenerating the NADH, retaining it in negatively charged nanofiltration membranes used in an enzyme reactor. In this system, the substrate was converted at concentrations of 300 g/L xylose, with a 96% yield and xylitol productivity of 3.33 g/L·h, and a kilo unit of XR was maintained for a 150 h reaction time with only a single dosage of NADH. The authors concluded that XR from *C. tenuis* seems to be well suited for enzymatic synthesis of xylitol due to its high NADH-linked activity, long-term stability, and the absence of strong product inhibition.

Neuhauser et al. (1998) reported on the *C. tenuis* XR-mediated NADH-dependent xylose reduction coupled with formate dehydrogenase (FDH) from *C. boidinii* for byproduct-free recycling of NADH used in a pH-controlled enzyme reactor. In this process, a fed-batch conversion of 0.5 M xylose to xylitol using yeast XR produced 2.8 g/L·h, which was a three-fold improvement when contrasted to a traditional batch reaction that employed equal initial concentrations of xylose and formate. Park et al. (2005) studied the production of xylitol from xylose by crude XR of *Candida peltata* in an electrochemical bioreactor. This bioreactor contains a graphite-Mn(IV) electrode (cathode) as a catalyst for the reduction of NAD^+ to NADH. Simultaneously, the resulting NADH is used to further reduction of xylose to xylitol.

Xylitol (3.5 mM) was generated from 10 mM xylose in the electrochemical bioreactor, while 1.5 mM of xylitol was produced in the conventional bioreactor. The xylose-to-xylitol reduction efficiency in the electrochemical bioreactor was twice as high as that found in the conventional bioreactor, which utilized NADH as a reducing equivalent. The enzymatic transformation of pure xylulose to xylitol has also been proposed by Hasumi et al. (1996). Synthesis of xylitol from xylulose using xylulose reductase by coupling the regeneration of NADH to the hydrogenase (from *Alcaligenes eutrophus*), with hydrogen gas as a reducing agent was reported. After a 34 h reaction period, 98% of the initial xylulose was converted into xylitol without byproduct formation. In the enzymatic approach, all experiments were carried out using a pure xylose-containing medium, and the process was optimized for this medium. In order to optimize the performance of the XR-catalyzed reactions for xylitol synthesis, the effects of process variables on productivity have been studied, including pH, temperature, initial substrate, and coenzyme concentration.

Effect of pH

The determination of the effect of pH on xylose reduction by monospecific XR (msXR) and dual specific XR (dsXR) was considered to be potentially valuable because of the pH-dependent ionization of the groups involved in substrate binding and catalysis (Nidetzky et al., 2003). In an enzymatic study leading to xylitol production, Yokoyama et al. (1995) reported that the optimum pH for the reduction of D-xylose by purified *Candida tropicalis* XR was 6.0, similar to the pH optima of most other XRs (Lee et al., 2003; Mayr et al., 2000; Neuhauser et al., 1997; Nidetzky et al., 2003; Ronzon et al., 2012; Verduyn et al., 1985a and Zeid et al., 2008). In another investigation, Lee et al. (2003) observed that the optimum pH for the conversion of D-xylose to xylitol by XR from *Candida parapsilosis* was 6.0, with 81 and 65% of maximum activity at pH 5.0 and 7.0, respectively. The optimum pH for xylitol oxidation was 8.0, with 85 and 68% of the maximum activity at pH 7.0 and 9.0, respectively. Maximal reductase activity at pH 6.0 and an alkaline pH optimum for xylitol oxidation (reverse reaction) are common features of similar XRs isolated from diverse microorganisms (Lee et al., 2003 and Lewis and Smith, 1967). Using NADH as coenzyme, the optimum pH for *Candida shehatae* XR activity was 6.5 (Wang et al., 2007). To determine the optimum pH range

for activity, *N. crassa* XR (NcXR) activity was measured at various pH values ranging from 3.5–8.0 at saturating concentrations of NADPH (0.2 mM) and xylose (1.0 M) prepared in universal buffer (Woodyer et al., 2005 and Zhao et al., 2009). The pH range for NcXR activity was 4.5–6.5, with an optimum value of about 5.5. The optimum pH of NcXR was slightly lower than that of the other XRs (pH 6.0) (Lee et al., 2003 and Zeid et al., 2008), but its profile is similar to many other XRs. The pH range for the production of xylitol by XR from *Pichia stipitis* was 5.0–8.0, with an optimum value of 6.0 (Verduyn et al., 1985a).

Effect of Temperature

An enzymatic method of xylitol production from D-xylose using isolated XR from *C. pelliculosa* was reported by Kitpreechavanich et al. (1984) in 100 mM Tris/HCl buffer (pH 7.5) at 35 °C, with a yield of 90% after 24 h incubation. In another experiment, Verduyn et al. (1985a) observed that the optimum temperature for XR from *P. stipitis* was 38 °C and that the NADPH-linked XR activity increased linearly from 20 °C to 38 °C. Nidetzky et al. (1996) reported the enzymatic synthesis of xylitol carried out in 50 mM Tris/HCl buffer (pH 7.0) at 25–35 °C in which the optimum operational temperature was 25 °C, which is similar to the temperature optimum in other experiments on the enzymatic conversion of xylose to xylitol (Neuhauser et al., 1998; Yokoyama et al., 1995 and Zeid et al., 2008). The optimum temperature for XR from *C. tenuis* under standard assay conditions was found to be 50 °C by Neuhauser et al. (1997). The NAD(P)H-dependent XR activity increased linearly from 25–50 °C, resulting in 4.5-fold activation of XR. Recently, Wang et al. (2007) pointed out that the optimum temperature for *C. shehatae* XR activity was between 35 and 40 °C. Woodyer et al. (2005) determined the optimum temperature for NcXR by assaying XR activities at temperatures ranging from 13–65 °C and reported the optimum value to be between 45 and 55 °C. The enzyme is rapidly inactivated at higher temperatures, whereas at lower temperatures, the reaction rate decreases with temperature according to the Arrhenius equation.

Effect of Substrate and Coenzyme Concentration

The activity of XR was measured spectrophotometrically at 340 nm by monitoring the oxidation of NADPH at substrate concentrations of 0.5 M xylose and 3.4 mM NADPH (Yokoyama et al., 1995). Nidetzky et al. (1996) synthesized xylitol from substrate mixtures of D-xylose and D-glucose (0.3–1.0 M each) or of D-xylose alone by XR in a charged membrane rector. Substrate concentrations in the range of 0.1–0.2 M could be completely converted to xylitol in one batch cycle. When more concentrated sugar solutions (0.3–1.0 M D-xylose and D-glucose each) or of D-xylose alone (0.6–2.0 M) were used, a complete substrate conversion could not be achieved. For enzymatic xylitol production, the initial xylose concentrations will typically be in a range of 0.5–1.0 M with 96% conversion using 0.1 mM coenzyme. The activities of XR were determined in a spectrophotometric test at 25 °C using the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.707 M D-xylose, and 0.22 mM NAD(P)H (Mayr et al., 2000, and Neuhauser et al., 1998). Rizzi et al. (1988) reported that the activities of *Pichia stipitis* XR were determined spectrophotometrically at 340 nm using the reaction mixture containing 250 mM xylose or xylitol, 0.115 mM $NAD(P)^+$ or NAD(P)H, 100 mM Tris/HC1 buffer (pH 7.0), and sufficient enzyme to produce changes in absorbancy of 0.05–0.15 per min. Substrate inhibition was not observed at substrate concentrations of 0.5 M xylose and 0.5 mM NAD(P)H (Rizzi et al., 1988; Verduyn et al., 1985a and Zhao et al., 1998). Product inhibition by xylitol was also not observed up to 0.5 M concentration (Verduyn et al., 1985a).

Since enzyme and NAD(P)H are expensive, the XR-catalyzed reduction of xylose requires reduction of enzyme costs and regeneration of coenzymes to make the enzymatic approach economically viable. The costs of intracellular XR preparation are tightly associated with the productivity of enzyme-producing microbial strains, media composition and process control. The reduction of enzyme costs through the generation of high-yield strains can have a substantial positive impact on industrial production costs. It is important to develop new and/or engineered strains that show higher XR production in the hemicellulosic hydrolysate. A simplified growth medium containing hydrolysate as a source of xylose, instead of commercial xylose, proves to be a cost effective substrate for XR production. Thus, the optimization of XR production from

robust efficient organisms using hydrolysate as an alternative carbon source can help to reduce the costs of enzyme preparation. One of the major drawbacks in the bioprocess utilizing hydrolysate is the inhibitory effect of some compounds derived during acid hydrolysis of LCM (Marton et al., 2006). In enzymatic xylitol production, the problems related to reaction inhibition can be overcome by treatment of the hydrolysate prior to reaction or by the hydrolysis of LCM with enzymes to produce an inhibitor-free hydrolysate. However, it should be kept in mind that downstream processing costs are usually major contributors to overall production costs. Thus, the innovative enzymatic approach can be very convenient and practical for use in large-scale production of xylitol because of the approach and simplicity of downstream processing.

2.8 XYLOSE REDUCTASE ENZYME

Xylose reductase (XR; EC 1.1.1.21) is an intracellular enzyme commonly found in yeast and filamentous fungi, often in several isozyme forms in the same species. This enzyme occurs in the cytoplasm of microorganisms, where it catalyzes the first step of D-xylose metabolism by reducing xylose to xylitol with the concomitant oxidation of NAD(P)H to $NAD(P)^+$ (Ronzon et al., 2012; Woodyer et al., 2005 and Zhao et al., 2009). XR has potential biotechnological application at least in two areas: xylose fermentation for bioethanol production and conversion of xylose to xylitol (Rawat and Rao, 1996 and Zhao et al., 2009). Despite its reported downstream processing to separate from yeasts and some potential uses in several sectors, the enzyme XR is not yet commercially available (Rawat and Rao, 1996; Ronzon et al., 2012 and Tomotani et al., 2009). To exploit its maximal potency on the conversion of xylose to xylitol, it is necessary to isolate and characterize XR from the potential xylose-fermenting yeast. In order to understand the relative roles of XR in xylose conversion, the functional properties of it must be known in detail.

2.8.1 XR Production and Activity Assay

XR enzyme is isolated from xylose-fermenting microbes (mainly yeast) by different methods such as ultrasonication, glass beads, sea sand and freeze-thaw treatment. Webb and Lee (1991, 1992) cultivated *Pichia stipitis* NRC 2548 in yeast

nitrogen base (YNB) medium and harvested the cells during late exponential phase (18–20 h) by centrifugation. They washed the cell pellet with ice-cold 0.25 M potassium phosphate buffer (pH 7.0), resuspended it in sufficient volume of the same buffer containing 5 mM EDTA to form a thin paste and added about 1.5 g of alumina to it. The cell suspension was subjected to 12–15 bursts of sonication to break the cells. The resulting cell debris and fragments were removed by centrifugation, and the supernatant was further clarified by centrifugation. The clarified supernatant was applied as a crude enzyme solution for activity assay. In another study (Rawat and Rao 1996), the cell mass of *Neurospora crassa* was harvested after 48 h of cultivation and rinsed with 0.5 M sodium phosphate buffer (pH 7.5). The cell pellet (10 g wet wt) was suspended in chilled Na-phosphate buffer containing 2 mM each of PMSF and EDTA. Cell free extract (CFE) was prepared by disrupting the cells through ultrasonication, followed by centrifugation, to remove the cell debris. XR enzyme extracted from yeast strain *Candida guilliermondii* FTI 20037, which has been cultivated in the fermentation medium (pH 5.5) formulated with D-xylose (18.8–50 g/L) for 34–50 h at 30 ºC and an agitation of 200 rpm (Rodrigues et al., 2006; Sene et al., 2001 and Tomotani et al., 2009). The cells were pelleted at the end of the fermentation period, washed twice with 0.1 M K-phosphate buffer (pH 7.2) and resuspended (68 g/L cell) in the same buffer. The cell suspension was subjected to disruption by 20-kHz sonication in 1 sec pulses for a period of 35–45 min at 4 ºC. The cell fragments were eliminated by centrifugation, and the supernatant was used as a source of XR for enzymatic analysis.

Different yeast cells were grown in yeast extract peptone dextrose (YPD) medium and sedimented at the end of the exponential growth phase (about 24 h of growth) by centrifugation, washed twice with chilled K-phosphate buffer (0.01–0.5 M; pH 7.0–7.6). One gram of the pellet was suspended in 2 mL phosphate buffer and homogenized with 3 mg of glass beads in a homogenizer operated at 2000 rpm for 7–10 min at 4 ºC. The cell debris was removed by ultracentrifugation, and the clear supernatant was employed for enzyme activity assays (Kuhn et al., 1995; Rizzi et al., 1988 and Nidetzky et al., 1996). The yeast *Candida tropicalis* IF0 0618 (ATCC 96745) was grown in YPD medium contained D-xylose (3% w/v) at 30 °C for 24 h at 130 rpm. The cells were harvested by centrifugation, and washed with sterile water once and 0.5 M K-phosphate buffer (pH 7.5) twice. The washed cell pellet was suspended in the

same buffer (cell and buffer ratio of 1:2–2.5 (w/v)) and then disrupted with sterilized sea sand in a mortar at 4 ºC. The resulting cell debris was discarded by centrifugation, and the supernatant fluid achieved was used as CFE for enzyme assays (Rangaswamy and Agblevor, 2002 and Yokoyama et al., 1995). Recently, Tamburini et al. (2010) harvested *C. tropicalis* strain DSM 7524 from xylose fermentation broth by centrifugation and resuspended in the extraction buffer (50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM dithiothreitol and 0.5 mM EDTA). They extracted XR enzyme via permeabilization of cell suspension with 5 cycles of freeze- and-thaw treatment in liquid nitrogen and centrifuged to get supernatant for assaying the activity of enzyme.

Based on the literature available, it can be realized that ultrasonic cell disruption is one of the most studied and popular techniques in XR preparation because it is simple and effective. In all studies, XR activity was assayed spectrophotometrically at 340 nm by monitoring the oxidation of NADPH coupled with the reduction of xylose to xylitol at 25 ºC and pH 7.0. Stoichiometrically, one molecule of NADPH is consumed (oxidized) per molecule of xylose reduced. Thus, the oxidation of NADPH (measured by a decrease in absorbance at 340 nm) directly correlates with xylose reduction. One XR unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per min at room temperature and at pH 7.0 (Nidetzky et al., 1996; Park et al., 2005; Tamburini et al., 2010; Yokoyama et al., 1995 and Zeid et al., 2008).

2.8.2 General Properties of XR

Based on the sequence and structural similarities, yeast and fungal XR has been classified as a member of the aldo-keto reductase (AKR) superfamily and more specifically, it belongs to the aldose reductase family (EC 1.1.1.21) of enzymes (Zhao et al., 2009). Yeast XR can be further divided into two groups according to coenzyme specificity: a group of NADPH-specific reductases known as monospecific XR (msXR) and another enzyme group active with both NADPH and NADH called dual specific XR (dsXR). dsXR is found in most yeast taxae known to ferment xylose such as *Candida shehatae*, *C. tenuis*, *Pichia stipitis*, and *Pachysolen tannophilus*. Both msXR and dsXR are homodimers composed of identical subunits of about 36 kDa molecular mass (Nidetzky et al., 2003). Mayr et al. (2000) reported that XR from yeast shows a

quite large structural and functional variability. It can be dimers or monomers, but most of the XR function as noncooperative, tightly associated dimers with a subunit molecular mass of 33–40 kDa (Lee et al., 2003). According to initial velocity and product inhibition studies, the *P. stipitis* (Rizzi et al., 1988) and *N. crassa* (Rawat and Rao, 1996) XR have been shown to catalyze the forward reaction via an 'iso-ordered bi-bi' kinetic mechanism, with the coenzyme binding first to the enzyme followed by the substrate and finally isomerisation of a stable enzyme form.

The enzyme XR [other terms: aldose or aldehyde reductase; alditol: $NAD(P)^+$ 1-oxidoreductase] has been isolated from *C. tropicalis* IFO 0618 by Yokoyama et al. (1995). Three isoforms (XR1, XR2, and XR3) of NADPH-dependent XRs have been purified and characterized. They had respective K_m values of 37, 30, and 34 mM for D-xylose and 14, 18, and 9 µM for NADPH but NADH did not act as a cofactor. However, NADH dependent activities of XR have been measured from cell free extracts (Horitsu et al., 1992). The specificities of the three XRs for several aldoses (DL-glyceraldehyde, L-arabinose, and D-xylose) were essentially the same. Both XR1 and XR2 were dimers composed of two identical subunits, and they had a molecular weight of 36 kDa. The pI values of XR1 and XR2 were 4.15 and 4.10, respectively, showing that some difference in charge exists between them. The optimal pH for both XRl and XR2 activities was around pH 6.0, though XR2 showed higher activity over a wider pH range than XRl did. Treatment of purified XRl and XR2 for 1 h at various temperatures resulted in the progressive loss of enzyme activities and the activities of both isoforms being completely lost after 1 h at 60 °C (Yokoyama et al., 1995). In addition, XR has been purified from *C. shehatae* (Ho et al., 1990), *C. tenuis* (Neuhauser et al., 1997), and *C. intermedia* (Mayr et al, 2000). Ho et al. (1990) concluded that this enzyme is remarkably stable at room temperature and 4 °C. The enzymatic synthesis of xylitol had been carried out at pH about 7.0 at 25 °C in 20 mL bioreactor. Fed-batch conversion of 0.5 M (76 g/L) xylose into xylitol yielded productivity of 2.8 g/L·h during 20 h (Neuhauser et al., 1998). The optimal conditions for the production of xylitol from xylose using XR are: temperature 20–38 °C (optimum 38 °C), pH values 5.0–8.0 (optimum pH 6.0), and xylose concentrations up to 0.5 M (Verduyn et al., 1985a).

2.9 OPTIMIZATION STRATEGIES

Optimization is referred to as a way to improve the performance of a process, a product or a system for achieving the maximum benefit from it. Generally, the term 'optimization' has been used in analytical chemistry as a means of discovering conditions applicable to a procedure that generates the best possible response (Bezerra et al., 2008). In order to scale-up the biochemical xylitol production, the process should be optimized. Commonly used optimization techniques are briefly described below:

2.9.1 One-Factor-At-a-Time Approach

Classically, optimization in analytical chemistry has been carried out by monitoring the influence of one variable at a time on an experimental response. While only one process variable is changed, others are maintained at a constant value. This optimization technique is called one-factor-at-a-time (OFAT) (Bezerra et al., 2008 and Jimbat, 2006). According to Montgomery (2001), OFAT method is the most extensively used experimental strategy for process optimization. This approach consists of selecting a starting point or baseline set of levels, for each factor, then successively changing each factor over its range with the other factors kept constant at the baseline level. Recently, Bari et al. (2009) reported that OFAT is used to determine the possible optimum level of parameters for further optimization of citric acid bioproduction from oil palm empty fruit bunches by central composite design (CCD) under response surface methodology (RSM). Literature survey revealed that sequential studies with OFAT method and RSM have not been followed in most of the optimization studies for the improvement of xylose and/or xylitol production (Canettieri et al., 2007; Marton et al., 2006; Roberto et al., 2003 and Silva and Roberto, 2001a). It is, therefore, necessary to employ a sequential optimization studies involving OFAT approach and statistical design for the improvement of xylitol production from MWS by XR enzyme.

2.9.2 Response Surface Methodology

To detect the effects of interaction among the variables, the optimization of production process, has been carried out using multivariate statistical techniques.

Among the multivariate techniques, response surface methodology (RSM) is the most relevant method used in optimization studies (Bezerra et al., 2008). RSM is defined as a combination of statistical and mathematical techniques useful for developing, improving and optimizing process (Sharma et al., 2009). Its main advantage is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Karacan et al., 2007). Furthermore, RSM can be well applied while a response or a set of responses of interest are influenced by several variables. The purpose is to simultaneously optimize the levels of these variables to achieve the best process performance (Bezerra et al., 2008). Numerous studies presented the use of RSM for optimization of process parameters (e.g., temperature, acid concentration and reaction time) for xylose recovery from LCM (Rahman et al., 2007 and Roberto et al., 2003) or microbial production of xylitol from lignocellulosic hydrolysate (Marton et al., 2006; Mussatto and Roberto, 2008 and Silva and Roberto, 2001a). To the best of my knowledge, no study has yet been conducted that uses RSM for optimization of enzymatic xylitol production from lignocellulosic hydrolysate. Hence, in this study, RSM has been considered as an important tool to optimize the process parameters for enzymatic conversion of xylose to xylitol from LCM hydrolysate.

2.10 CONCLUSION

Xylitol is a pentahydroxy sugar alcohol and is used not only as a functional sweetener but also as a platform chemical for the manufacture of industrially important other chemical products. Commercially, xylitol is manufactured by chemical process with a yield of 50–60%, which has some disadvantages such as a high energy requirement, extensive separation and purification that results in elevated product cost. Yeasts are considered as the best xylitol producers among the microorganisms studied. Enhancement in productivity and yield is important factors in industrial xylitol production. Taking into account the limitation of microbial production of xylitol, especially the necessity of huge volume of sterile distilled water and long residence time, it is important to emphasize on the development of XR-dependent bioconversion of xylose from hemicellulosic hydrolysate. Although the yield of microbial reduction of xylose to xylitol could be improved (by 65–85 and 86–100%) using different production methods with natural and transformed yeasts, the chemical process would still be very

competitive in terms of industrial-scale production. However, the fermentation method has not yet been able to accumulate the advantages of the chemical process due to the low initial xylose concentration used as well as to the downstream processing problem regarding the media ingredients.

The production of xylitol from xylose by XR is an attractive alternative to chemical and microbial processes. The XR enzyme is not available commercially despite its potential applications in the synthesis of xylitol and/or ethanol. To explore the maximal efficacy of XR on xylose to xylitol conversion, it is necessary to isolate and characterize the XR from the potential xylose-fermenting yeast. Compared to the microbial processes, the enzymatic approach to xylitol synthesis is expected to achieve a substantial increase in productivity as mass transfer limitations are overcome in an enzyme reactor. One significant advantage of *in vitro* enzyme-based xylitol production is that it can afford an easy recovery of xylitol. It has been reported that XR-catalyzed reduction of D-xylose is NAD(P)H-dependent, and the conversion of D-xylose into xylitol is higher than 95% (Neuhauser et al., 1998 and Nidetzky et al., 1996). So far, enzymatic approaches have been optimized using a pure xylose-containing medium.

Use of XR to produce xylitol from LCM derived xylose is not yet reported. This technology might offer a bright prospect in the biorefinery industry. Hence, much research is required to develop efficient and bioconversion friendly hydrolysis methods for LCM, robust microorganisms to prepare XR in a cost effective way, and methods for an optimized enzyme-based xylitol production. The enzymatic approach might be able to overcome the disadvantages of the largely used chemical process and the fermentation process under investigation.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This research was undertaken to characterize *Meranti* wood sawdust (MWS) as a raw material for xylose production and an enzyme xylose reductase (XR) for xylitol production as well as to optimize processes for xylose recovery and xylitol production. This study comprised of four phases: MWS characterization, xylose recovery, XR preparation, and xylitol synthesis. This chapter outlines the experimental procedures for the characterization of MWS, recovery of xylose from MWS, preparation of XR from *Candida tropicalis*, and synthesis of xylitol from xylose. A flow diagram summarizing the overall experimental approach for enzymatic synthesis of xylitol is shown in Figure 3.1.

3.2 MATERIALS

3.2.1 Chemicals and Reagents

All the chemicals used in the current study were of analytical grade and purchased from various suppliers. A list of chemicals used in this study is given in Appendix A1 (Table A.1). Ultrapure water was used to prepare various solutions and reagents.

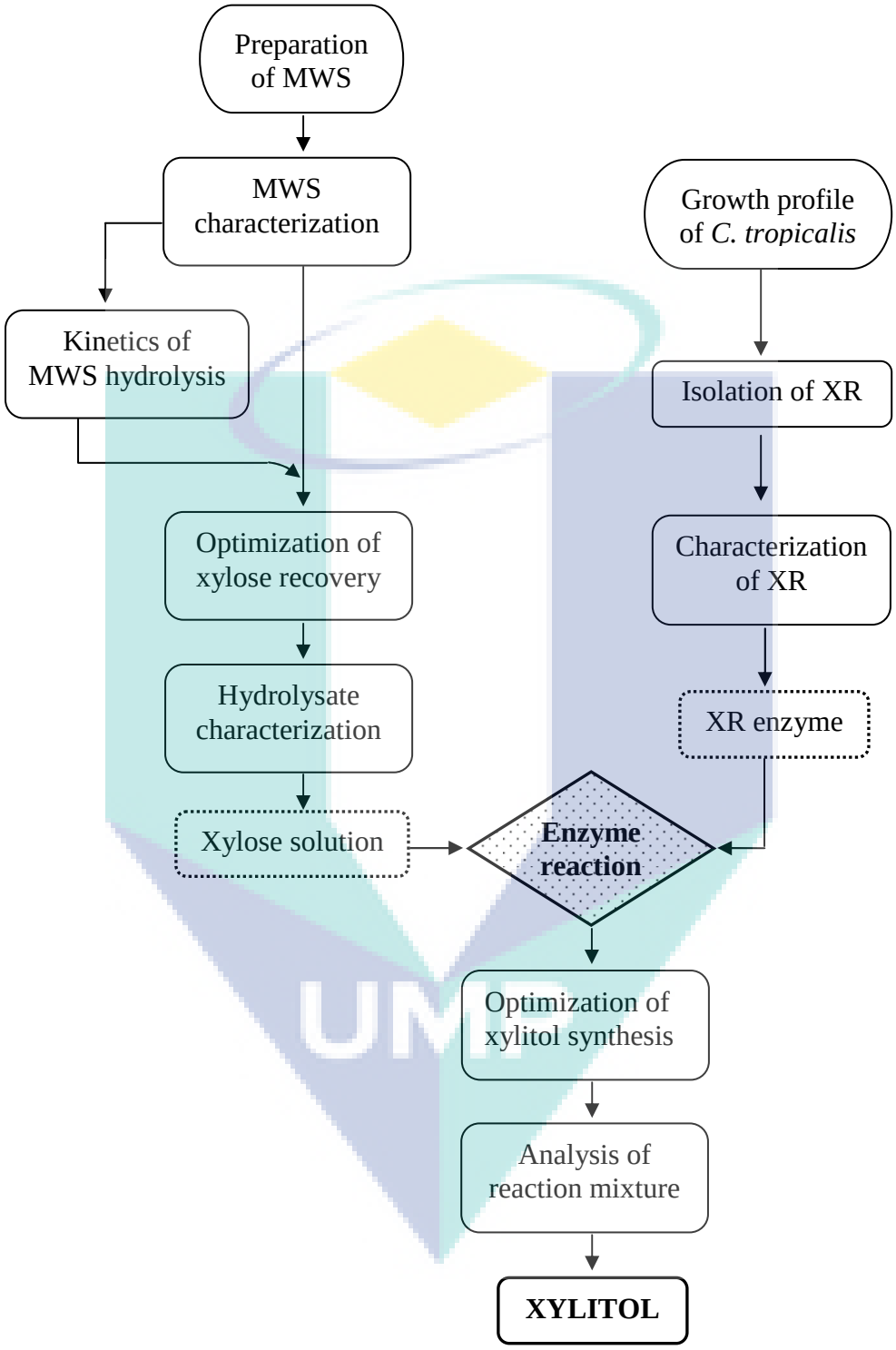


Figure 3.1: Experimental flowchart for enzymatic synthesis of xylitol from MWS

3.2.2 *Meranti* Wood Sawdust (MWS)

The raw material, *Meranti* wood sawdust (MWS), used throughout this study was collected from Seng Peng Sawmills Sdn Bhd at Gambang, Kuantan, Malaysia. The MWS was initially screened to remove oversized particles, sun dried and then stored at room temperature for further use.

3.2.3 Microbial Strain

The microorganism used in this study was *Candida tropicalis* IFO 0618 (ATCC® 96745™), a potential xylose-fermenting yeast. It was obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA.

3.2.4 Microbiological Media

YPD Medium

The yeast extract peptone dextrose (YPD) medium was used for the growth and maintenance of *C. tropicalis* IFO 0618. It is often called conservation or storage medium having the following composition as formulated by Yokoyama et al. (1995): 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, and 0.5% $MgSO_4 \cdot 7H_2O$ (w/v) in ultrapure water. The pH was adjusted to 6.0 with 1 M HC1 before autoclaving at 120 ºC and 15 psi for 20 min. The recipe of YPD agar medium was similar to that of the YPD medium except that 2% (w/v) agar was added as the gelling agent.

Synthetic Growth Medium

The synthetic growth medium was utilized for cultivating the yeast *C. tropicalis* IFO 0618. This medium composed of 3% xylose, 0.3% yeast extract, 0.3% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$ (w/v) in ultrapure water (Yokoyama et al., 1995). The pH of the medium was adjusted to 6.0 and then autoclaved as mentioned above in YPD medium. To prevent undesired reactions, the carbon sources (such as glucose or xylose), were dissolved in distilled water and autoclaved separately from other medium

ingredients. The sterilized carbon sources were then mixed together with other medium components before use.

YP-hydrolysate Medium

YP-hydrolysate medium was used for the growth and maintenance of adapted yeast strain. This medium was prepared from *Meranti* wood sawdust hemicellulosic hydrolysate (MWSHH) containing 1.88% xylose instead of using commercial glucose solution (2%). The rest of the media ingredients were the same as the YPD medium (i.e., 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, and 0.5% $MgSO_4 \cdot 7H_2O$ (w/v)). The preparation of YP-hydrolysate agar medium was similar to that of the YP-hydrolysate medium except that 2% (w/v) agar was utilized. The pH of these media was adjusted to 6.0 with 1 M HC1 before autoclaving at 120 ºC and 15 psi for 20 min.

Hydrolysate Growth Medium

Hydrolysate growth medium was prepared with raw MWSHH containing 1.88% xylose. The required amount of pure xylose and the other components (i.e., 0.3% yeast extract, 0.3% K_2HPO_4 and 0.1% $MgSO_4 \cdot 7H_2O$) were added to yield the same composition formulated for synthetic growth medium. The pH of the medium was adjusted at 6.0 with 1 M HCl and autoclaved at 120 ºC for 20 min.

3.3 OPERATIONAL FRAMEWORK

Figure 3.2 presents the schematic diagram summarizing the main experimental procedures of this study. The total experimental methodology is detailed below in four phases.

3.3.1 Phase 1: Characterization of MWS

The screened MWS (particle size <0.5 mm) was oven dried and used to characterize its prime structural components. The analyses of MWS biomass composition are very important to assess the reactivity of components in the biomass.

The MWS was analyzed for its cellulose, hemicellulose, xylan, lignin, extractives, and ash content following the standard procedure.

3.3.2 Phase 2: Recovery of Xylose

This phase describes the recovery of xylose from MWS by acid hydrolysis. There are three steps in this phase as detailed below:

(i) The first step involved the study on the effect of residence time, temperature, acid concentration, and liquid to solid ratio (LSR) on the formation of xylose and byproducts from MWS. The effective levels of process factors were selected through one-factor-at-a-time (OFAT) method.

(ii) In the second step, Saeman's kinetic model (Saeman, 1945) was adopted to determine the kinetic parameters (rate constants) to predict the concentration of the major compounds released during acid hydrolysis of MSW.

(iii) The third step involved the optimization of the MWS hydrolysis by applying RSM and the determination of the influences of temperature, H_2SO_4 concentration and residence time (found responsible, as determined by OFAT), as well as the formation of undesired products (glucose, arabinose, acetic acid, furfural, HMF, and LDPs).

3.3.3 Phase 3: Preparation of XR

Xylose reductase (XR) was prepared from the yeast *Candida tropicalis* for bioconversion of xylose, extracted from MWS, to xylitol in a batch system. The isolated XR was characterized based on enzyme activity, stability, kinetic constants, and minimum inhibitory concentration (MIC) of byproducts on XR.

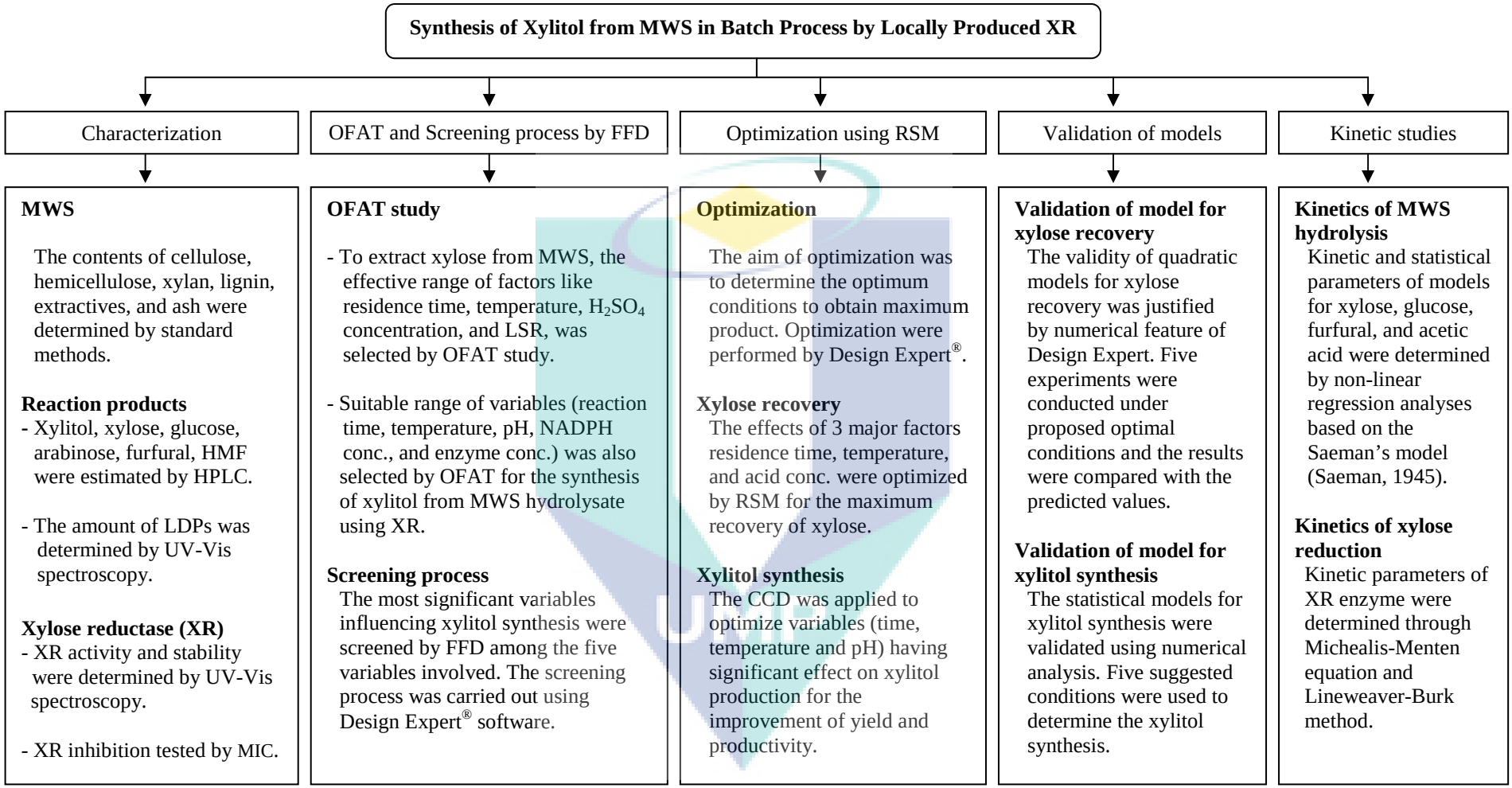


Figure 3.2: Schematic diagram of operational framework of this study

3.3.4 Phase 4: Synthesis of Xylitol

In this phase, enzymatic synthesis of xylitol from MWS was conducted in the following three steps:

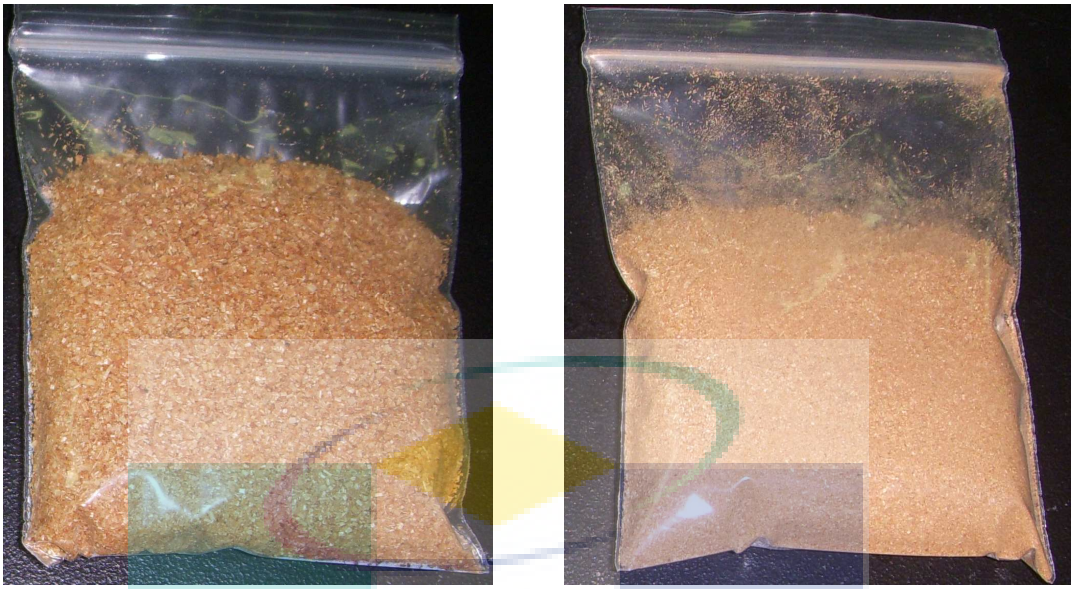
(i) The suitable range of variables that showed effects on xylitol bioproduction from MWS hemicellulosic hydrolysate (MWSHH) using XR were chosen by single factor experiment.

(ii) The significant factors influencing xylitol synthesis were identified. Factorial design was employed to define the important factors and to determine the interrelationship among reaction time, temperature, pH, NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) concentration, and enzyme concentration in enhancing xylitol yield.

(iii) The optimization of the xylitol production process was carried out and the interactions among the most significant variables (reaction time, temperature and pH) that governed the production of xylitol were determined by RSM.

3.4 MWS SAMPLE PREPARATION

The dried MWS was passed through a 0.5 mm sieve using a vibratory sieve shaker (Analysette 3 Pro, Fritsch, Germany) to select the particle size less than 0.5 mm (>0.5 mm retained). The screened MWS was homogenized, in a single lot, in order to avoid compositional differences among aliquots and stored in polypropylene bags at ambient temperature until further use. Figure 3.3 shows the appearance of the raw and screened (particle size <0.5 mm) MWS. Aliquots from the homogenized MWS were oven dried at 105 ºC for 12 h, cooled in desiccator for 30 min and then subjected to analysis or experiments. The methods applied to characterize MWS are detailed in the following section.



(a) (b)

Figure 3.3: Photographs representing the (a) raw and (b) screened MWS

3.5 CHARACTERIZATION OF MWS

MWS characterization was performed via chemical analyses of its structural components. The major constituents of MWS biomass were analyzed through standard methods, which are addressed below:

3.5.1 Determination of Extractives

In plant cell wall, the extractives are a group of compounds principally consisting of fats, fatty acids, fatty alcohols, terpenes, phenols, steroids, resin acids, waxes, etc. (Han and Rowell, 1997). The extractives in the MWS were determined as outlined in TAPPI (Technical Association of Pulp and Paper Industry) standard method (TAPPI T 204 cm-97, 2007). Two g of oven dried MWS sample was weighed in extraction thimbles (Whatman) in duplicate. The clean round bottom flask (RBF) was dried in oven at 105 ºC for 1 h, and its weight was recorded as RBF_1 when cooled to room temperature. Ethanol-toluene solution was made by mixing one volume of ethanol with two volumes of toluene and 160 mL of this solution was filled in the preweighed 500 mL RBF. The two thimbles were inserted in the Soxhlet apparatus (Favorit®) and connected the RBF at the bottom. The water flow was turned on to allow the gas

evaporated to condense, and the extraction was carried out. After 8 h of extraction, the ethanol-toluene solution in the RBF was dried out using digital water bath. Finally, the RBF was dried in oven at 105 ºC for overnight. The RBF was placed in desiccator for 30 min, and the weight of the RBF plus dry extractives (i.e., ethanol-toluene solubles) was recorded as RBF_2. On the other hand, the solid residues retained in the thimbles were oven dried at 105 ºC for 12 h and the resulting dried residues were named as extractive-free sample (EFS). The EFS was kept in desiccator and then analyzed for holocellulose, alpha (α)-cellulose, and lignin content in the MWS. The percentage of extractives was calculated based on Eq. (3.1).

$$\text{Extractives}\,(\%) = \frac{RBF_2 - RBF_1}{\text{Oven dry weight of MWS}} \times 100 \qquad (3.1)$$

Where, RBF_1 = dry weight of RBF and RBF_2 = dry weight of RBF + dry extractives.

3.5.2 Determination of Holocellulose

Holocellulose is a biopolymer of plant materials and is defined as the combination of cellulose and hemicellulose. It usually accounts for 65–70% of the plant dry weight. The preparation and estimation of holocellulose from EFS were conducted following a standard method as outlined by Han and Rowell (Han and Rowell, 1997). Two g of oven dried EFS (WX) obtained from ethanol-toluene test (as addressed in subsection 3.5.1) was transferred from the thimble into a 250 mL glass beaker. To the beaker, 100 mL of distilled water, 1.5 g sodium chloride and 5 mL of 10% acetic acid were added and mixed with a glass rod. The beaker was heated in a boiling water bath. After 30 min, 1.5 g NaCl was added, and after 30 min, 5 mL of acetic acid (10%) was added and mixed. This step was repeated until 6 g of NaCl has been added with mixing. The mixture was heated for another 30 min after the final NaCl was added. At the end of 3 h reaction, mixture was cooled in an iced water bath and then vacuum filtered through a preheated and preweighed glass filtering crucible with porosity 1 (Simax, Czech Republic) and recorded as Z_1. The residue on the filter was rinsed with ice cold water and then with acetone until it became white in color (the color of holocellulose is white). The crucible was left in an oven at 105 ºC for a day, placed in desiccator for 30

min and then weighed as final weight of the crucible plus dry residues (Z_2). The content of holocellulose was determined by Eq. (3.2).

$$\text{Holocellulose content (\%)} = \frac{Z_2 - Z_1}{WX} \times 100 \quad (3.2)$$

Where,

Z_1 = Crucible's dry weight

Z_2 = Crucible's dry weight + residues dry weight

WX = EFS's dry weight from ethanol-toluene extraction

3.5.3 Determination of α-Cellulose

The determination of α-cellulose is a continuous approach from procedure 3.5.2 in pursuit of the ultimate pure form of wood fiber. Extractive-free holocellulose is treated with sodium hydroxide and then with acetic acid, resulting in the solid residue defined as α-cellulose. Hence, the last fraction provides the hemicellulose content (Han and Rowell, 1997). The α-cellulose content was determined following a standard method (TAPPI T 203 os-74, 1994). The oven dried residue from the holocellulose sample was transferred into a 250 mL beaker. 17.5% (w/v) sodium hydroxide solution was prepared. Initially, 15 mL of the NaOH solution was added in the beaker and covered with a watch glass, and then stirred for 1 min at ambient temperature. Then 10 mL more of the NaOH was added and stirred for 45 sec. To the reaction mixture, 10 mL of NaOH was again added and stirred for 15 sec. After standing for 3 min (total time now is 5 min), 10 mL of NaOH, was added and stirred for 2.5 min. This step is repeated for 4 times and allowed to stand for 30 min. After a total of 45 min reaction, 100 mL of distilled water was added, stirred and left for 30 min (total time now is 75 min). The sample was vacuum filtered with a filtering crucible (porosity 3, Simax) of known dry weight (A_1), which was fitted with glass wool. The sample was washed with 8.3% NaOH, followed by 650 mL distilled water. The suction was stopped to fill the crucible with 2 N acetic acid and left for 5 min. Finally, the residue was rinsed with distilled water until the acid smell was gone. The filtering crucible with residue was oven dried

at 105 ºC for overnight, weighed and recorded as A_2. Alpha-cellulose content was calculated through Eq. (3.3).

$$\alpha\text{-Cellulose content}(\%) = \frac{A_2 - A_1}{WX} \times 100 \quad (3.3)$$

Where, A_1 = Crucible's dry weight

A_2 = Crucible's dry weight + residues dry weight

WX = EFS's dry weight from ethanol-toluene extraction

3.5.4 Determination of Hemicellulose

The hemicellulose content of MWS biomass was calculated from the values of holocellulose and alpha-cellulose from the Eq. (3.4) as reported by Han and Rowell (1997).

$$\text{Hemicellulose}(\%) = (\text{Holocellulose content}) - (\alpha\text{-Cellulose content}) \quad (3.4)$$

3.5.5 Determination of Xylan Content

The content of xylan in the MWS biomass was computed according to the Eq. (3.5) as also reported by Han and Rowell (1997).

$$\text{Xylan content}(\%) = (132/150) \times \text{Hemicellulose content} \quad (3.5)$$

Where, 132/150 is the stoichiometric factor between xylan and hemicellulose.

3.5.6 Determination of Lignin (Acid-Insoluble)

Acid-insoluble lignin is also known as "Klason lignin", defined as a constituent of wood or pulp that is insoluble in 72% (w/w) sulfuric acid. It was determined according to the standard method (TAPPI T 222 om-02, 2002). One g of EFS (oven dried) was weighed out in 50 mL beaker. The beaker was placed in cold water bath and

15 mL of 72% H_2SO_4 was measured and poured slowly into the beaker while stirring the sample with a glass rod. After the material stirred and dispersed thoroughly, the beaker was covered with a watch glass and incubated in a water bath at 20 ºC for 2 h. During this time, the material was stirred every 20 min to ensure complete solution. About 400 mL of distilled water was heated in 2 L Erlenmeyer flask, and the material was carefully transferred from the beaker to the flask. The beaker was rinsed (two times) with hot distilled water, and the flask was filled with water until the volume become 575 mL, which results in a 3% solution of H_2SO_4 for secondary hydrolysis. The top of the flask was connected with a reflux condenser, and the mixture was boiled for 4 h on a hot plate. The flask was kept in an inclined position until the insoluble material (i.e., lignin) settled at the bottom. Without shaking the flask, the supernatant solution, was filtered through a glass filtering crucible (porosity 3) of known dry weight. Then the lignin precipitate was transferred quantitatively to the filter using hot water and a glass rod, and the flask was rinsed several times with hot water. The crucible with lignin residue was oven dried at 105 ºC for overnight to constant weight, cooled in desiccator and weighed. The lignin content in the test material was computed using Eq. (3.6).

$$\text{Lignin content (\%)} = \frac{\text{Dry weight of lignin residue}}{\text{WX}} \times (100 - \text{x}) \qquad (3.6)$$

Where, WX = EFS's dry weight from ethanol-toluene extraction (g) and x = percentage of extractives.

3.5.7 Determination of Ash Content

The ash content of wood fiber is usually referred to as the residue remaining after ignition. It indicates an approximate measure of the inorganic matter and mineral salts found in the fiber after combustion (Han and Rowell, 1997). The ash content of MWS was estimated according to the TAPPI standard method (TAPPI T 211 om-02, 2002). The porcelain crucible and its cover were cleaned and ignited at 800 ºC in a muffle furnace (Size 2, Gallenkamp, The Netherlands) for 1 h to constant weight. After ignition, the crucible with cover were cooled slightly and carefully placed in desiccator for 1 h to cool down to room temperature. The crucible and cover were weighed just

before analysis and recorded as initial weight (C_1). Five g of MWS (oven dried) were loaded in the crucible with known dry weight. The wood sample was burnt directly under low flame of Bunsen burner until it has been carbonized fully (as indicated by no smoke; only white residue left). The crucible with cover was placed in the preheated furnace at 800 ºC for 3 h to burn off all the carbon. At the end of ignition period, the crucibles were removed from the furnace and allowed them to cool somewhat. The crucibles were then put in desiccator to cool to room temperature. The crucible containing ash was reweighed and marked as C_2, and the percentage of ash in the MWS was calculated by Eq. (3.7).

$$\text{Percentage of ash} = \frac{C_2 - C_1}{\text{Oven dry weight of MWS}} \times 100 \quad (3.7)$$

Where, C_1 = dry weight of porcelain crucible; C_2 = dry weight of porcelain crucible + ash and $C_2 - C_1$ = weight of ash in MWS.

3.6 XYLOSE RECOVERY FROM MWS

3.6.1 Acid Hydrolysis of MWS

Three g of MWS on an oven dry basis (o.d.b.) was mixed with the required amount of sulfuric acid solution (%, w/w) in 250 mL screw capped Erlenmeyer flask. The slurries were stirred on a magnetic stirrer (EMS-HP-7000, ERLA) for 10 min at room temperature in order to equilibrate the acid concentration between the bulk liquid phase and biomass. Batch hydrolysis was performed in an autoclave (Hiclave HVE-50, Hirayama, Japan) under different experimental conditions (Parajó et al., 1995 and Zeid et al., 2008). The flasks were placed in the autoclave at room temperature and heated to achieve the desired temperature for desired length of time. After the residence time had elapsed, the autoclave was cooled down to 95 ºC, opened, and the flasks were collected. During hydrolysis, heating up and cooling down times showing pressures above the atmospheric pressure were not calculated. The liquid and solid phases were separated by filtration (Whatman no. 1 filter paper), and the filtrate was named as *Meranti* wood sawdust hemicellulosic hydrolysate (MWSHH). The solid residue was washed twice

with ultrapure water to recover residual sugars. The combined volumes of filtrate and washes were measured and recorded. The MWSHH was neutralized with calcium oxide (CaO) powder to a pH 6.0. After 1 h, the $CaSO_4$ precipitate formed was removed by filtration. The filtrate was stored at 4 ºC, and analyzed for xylose, glucose, arabinose, acetic acid, furfural, HMF, and LDPs. Unless otherwise stated, all the experiments were carried out in triplicate and average values with standard deviation (SD) were recorded.

3.6.2 Parameter Design for Xylose Recovery

The effects of residence time, temperature, H_2SO_4 concentration, and liquid to solid ratio (LSR) on the hydrolysis of MWS were examined with the one-factor-at-a-time (OFAT) method to design the optimum range of the parameters. The variation of process parameters employed for each study is shown in Table 3.1, which was chosen based on previous reports regarding acid hydrolysis of different lignocellulosic materials (LCMs) (Dominguez et al., 1997; Mussatto and Roberto, 2005; Nigam, 2001 and Silva et al., 1998). For the initial OFAT experiment, the levels of three factors out of four were held constant (temperature at 130 ºC, H_2SO_4 concentration at 2% (w/w), and LSR at 8 g/g) on the basis of previous research reports (Liaw et al., 2008 and Parajó et al., 1995). The first parameter was then varied until an optimum value was reached. This optimum value for the first factor was then used while the second parameter was varied and so on.

Table 3.1: Variation of process factors for MWS hydrolysis using OFAT

Study	Residence time (min)	Temperature (ºC)	H_2SO_4 conc. (%, w/w)	LSR (g/g)
Effect of residence time	10–120	130	2	8
Effect of temperature	60	105–130	2	8
Effect of H_2SO_4 conc.	60	125	2–12	8
Effect of LSR[a]	60	125	4	8–20

[a] LSR = liquid to solid ratio

3.6.3 Kinetic Models for MWS Hydrolysis

Oven dried MWS (3 g) was mixed with H_2SO_4 solution (%, w/w) at a LSR of 8 g/g. Hydrolysis was carried out at 130 ºC in media consisting of 2–6% (w/w) H_2SO_4, values were selected according to the previous research reports (Parajó et al., 1994 and Téllez-Luis et al., 2002). Samples were taken at various time intervals in the range of 0–120 min and the hemicellulosic hydrolysate was processed as mentioned in subsection 3.6.1. The hydrolysate was stored at 4 ºC and analyzed by HPLC for estimating xylose, glucose, furfural, and acetic acid that are involved in kinetic studies on MWS hydrolysis.

A variety of models for the acid hydrolysis of lignocellulosics are available in the literature (Carrasco and Roy, 1992; Garrote et al., 2001; Maloney et al., 1986 and Saeman, 1945). The simplest model for the hydrolysis of hemicellulose involves a series of pseudohomogeneous irreversible first-order reactions, which is represented as:

$$\text{Xylan(s)} \xrightarrow{k_1} \text{Xylose(aq)} \xrightarrow{k_2} \text{Decomposition products} \tag{3.8}$$

where k_1 and k_2 are the rate of xylose generation and decomposition (min^{-1}), respectively. Based on this model and solving differential equations, xylose concentration [X] as a function of time (t) can be written by the following scheme (Saeman, 1945):

$$[X] = \frac{k_1[Xn_p]}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) + [X_0]e^{-k_2 t} \tag{3.9}$$

The initial concentrations of xylose at a time t = 0 and xylan (expressed as potential concentration of xylose) are denoted by [X_0] and [Xn_p], respectively. Assuming [X_0] to be closely equal to 0, a simplification of the model leads to Eq. (3.10), which is called Saeman's model in this work. The potential concentration (PC) of xylose (g/L) can be calculated, and the kinetic constants k_1 and k_2 can be determined by regression fitting the kinetic model equation to the experimental data.

$$[X] = \frac{k_1[Xn_p]}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \tag{3.10}$$

The kinetic model concerning the hydrolysis of cellulose also involves a series of pseudohomogeneous irreversible first-order reactions. The decomposition reactions were negligible in this study as the operating conditions were not favorable to degradation of glucose; hence the model can be simplified by the following reaction scheme:

$$\text{Glucan (s)} \xrightarrow{k_3} \text{Glucose (aq)} \tag{3.11}$$

By solving differential equations, the glucose concentration [G] as a function of time can be expressed as Eq. (3.12).

$$[G] = [G_p](1 - e^{-k_3 t}) \tag{3.12}$$

where [G_p] is the potential concentration (PC) of glucose (g/L), which can be determined by regression analysis and k_3 is the rate of glucose generation (min^{-1}).

The kinetic model for furfural concentration [F] as a function of time can be expressed as

$$[F] = [F_p](1 - e^{-k_4 t}) \tag{3.13}$$

where k_4 is the furfural formation rate (min^{-1}) and [F_p] is the PC of furfural (g/L), which can be obtained by regression.

The model for acetic acid generation can be developed based on the release of acetyl groups from hemicellulose without further reactions, which can be represented as below:

$$\text{Acetyl groups} \xrightarrow{k_5} \text{Acetic acid} \tag{3.14}$$

The acetic acid concentration [A] in the hemicellulosic hydrolysate as a function of time can be expressed by Eq. (3.15).

$$[A] = [A_p](1 - e^{-k_5 t}) \tag{3.15}$$

where [A_p] is the PC of acetic acid (g/L), which can be estimated by regression analysis, and k_5 is the rate of acetic acid generation (min^{-1}). Eqs. (3.10), (3.12), (3.13), and (3.15) were employed to model the hydrolysis of MWS using sulfuric acid. Kinetic parameters and constants were obtained by iteration non-linear regression analyses, and the results were statistically evaluated.

3.6.4 Analysis of Data

All the hydrolysis experiments were run in triplicate and data were expressed in average values ± SD. For kinetic modeling of MWS acid hydrolysis, experimental data were fitted to the proposed model equations and non-linear regression analyses were executed following Newton's method (Solver, Microsoft Excel 2003, Microsoft Corporation, USA), by minimizing the sum of squares of residuals between actual and calculated values as reported earlier (Garrote et al., 2001). The modeling results were evaluated statistically with the determination coefficient R^2 to find out the reliability of the models.

3.6.5 Optimization of Xylose Recovery

The optimization study on xylose recovery was further continued with the central composite design (CCD) of response surface methodology (RSM) for xylose yield and selectivity as the dependent variables or responses of the design experiments. The RSM was used to optimize the hydrolysis process and to explore the interaction effects of the variables (Montgomery, 2001). The optimum levels of three major independent variables namely temperature, acid concentration, and residence time were chosen from the result of the OFAT study (subsection 5.2.1). Another factor LSR was set constant at 8 g/g throughout the investigation as it was found to be insignificant in the recovery of xylose. The CCD of experiments was carried out with a 2^3 full factorial

central composite design of combination variables at two coded levels (high, +1 and low, -1 level). This design consists of six axial (star) points corresponding to an α value of ±2 and six replication of the center points (coded level 0), resulting in a total of 20 sets of experimental runs. It is noted that α is the distance of the axial point from the center. The value of α (axial distance) was chosen to be ± 2 to make the design rotatable. Hydrolysis experiments were performed according to the procedure described above in subsection 3.6.1. The ranges and levels of the independent variables involved are given in Table 3.2. The second order model was selected for predicting the optimal point which is expressed as Eq. (3.16).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (3.16)$$

where Y represents predicted response variable; β_0 is the interception coefficient; β_1, β_2, and β_3 are the linear coefficients; β_{11}, β_{22}, and β_{33} are the quadratic coefficients; β_{12}, β_{13}, and β_{23} are the second order interaction coefficients and A, B, and C are the coded input variables studied (temperature, acid concentration, and residence time). The Design Expert® software version 6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to generate the experimental matrix and to optimize the regression equation (Eq. (3.16)). The statistical significance of the model equation and regression coefficients was determined by performing analysis of variance (ANOVA). This analysis included Fischer's test (F-test), its associated probability, determination coefficient (R^2) and correlation coefficient (R). The optimum values of the input variables were acquired by numerical analysis utilizing Design Expert program based on the criterion of desirability. Five sets of experiments were carried out under proposed optimal conditions to validate the CCD developed model. A confirmation experiment was also carried out to prove the model.

Table 3.2: Ranges and levels of independent variables involved in CCD for xylose recovery

Variables	Symbol	Range and levels				
		-α	-1	0	+1	+α
Temperature (ºC)	*A*	121	123	125	127	129
Acid concentration (%, w/w)	*B*	2	3	4	5	6
Residence time (min)	*C*	20	40	60	80	100

α = ± 2

3.7 PREPARATION AND CHARACRERIZATION OF XR

3.7.1 Maintenance of Microbial Strain

The yeast strain, *Candida tropicalis* IFO 0618, was incubated on YPD agar plate at 30 ºC for 30 h (Figure 3.4) and maintained at 4 ºC in a refrigerator for short term storage. Subculture of the strain was carried out once a month. The frozen stock cultures were maintained at –80 ºC in YPD medium containing 20% (w/v) sterilized glycerol in 2 mL screw capped vials for extended storage (up to 2 years). Prior to each experiment, cells were transferred from stock culture plate and grown at 30 ºC for 30 h on fresh Petri dishes containing YPD agar medium.

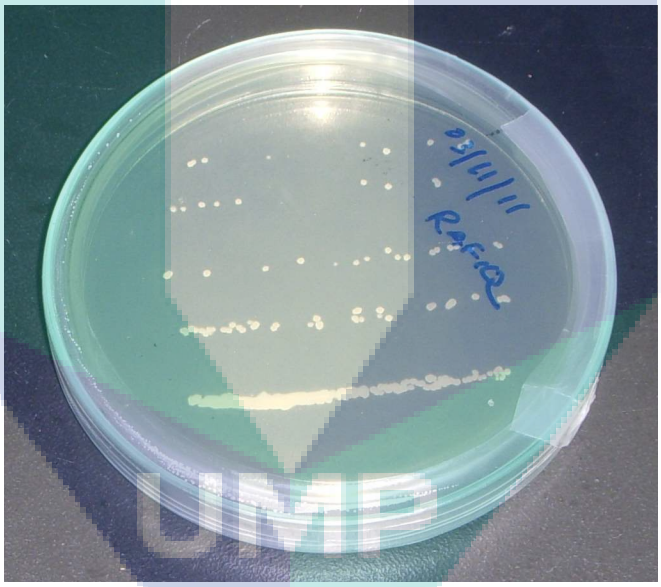


Figure 3.4: *C. tropicalis* grown on YPD agar plate at 30 ºC for 30 h

3.7.2 Preparation and Maintenance of Adapted Yeast Strain

The inoculum prepared in synthetic growth medium was further cultured in media containing MWS hemicellulosic hydrolysate (MWSHH). Adapted yeasts were prepared by sequentially transferring and growing cells in media containing increasing concentrations (10, 20, 40, 60, 80, and 100% (v/v)) of crude MWSHH (pH 6.0) supplemented with (%, w/v): required amount of xylose, 0.3 yeast extract, 0.3 K_2HPO_4 and 0.1 $MgSO_4 \cdot 7H_2O$ to yield the same composition used for synthetic growth medium

(as mentioned in subsection 3.2.4: synthetic growth medium) (Nigam, 2001 and Rao et al., 2006). The cells were collected by centrifugation at 4000 rpm for 10 min at 4 ºC (Eppendorf centrifuge 5810 R, Eppendorf, Germany) and then resuspended in fresh hydrolysate growth medium. Six successive batch cultures were carried out with hydrolysate media at 30 ºC for 24 h at 150 rpm in a rotary shaker incubator to obtain adapted yeast *C. tropicalis*. The adapted strains were maintained in agar plates made from YP-hydrolysate agar medium (subsection 3.2.4: YP-hydrolysate medium) and utilized in subsequent XR preparation experiments. Figure 3.5 depicts the culture of adapted *C. tropicalis* grown at 30 ºC for 36 h on YP-hydrolysate agar medium.

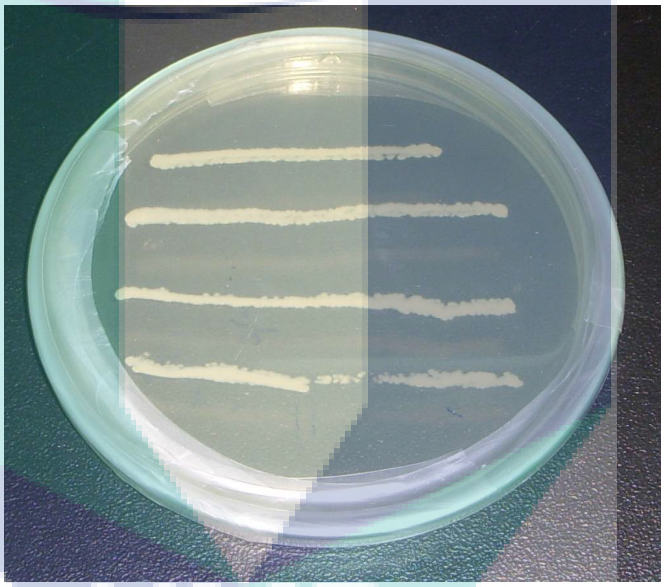


Figure 3.5: Adapted *C. tropicalis* grown on YP-hydrolysate agar medium at 30 ºC for 36 h

3.7.3 Inoculum Preparation and Growth Conditions

The inoculum was prepared from fresh culture grown at 30 ºC for 36 h on YP-hydrolysate agar plate by transferring a single colony of adapted *C. tropicalis* to a 250 mL Erlenmeyer flask containing 50 mL of hydrolysate growth medium as outlined by Yokoyama et al., 1995. The inoculated flask was incubated at 30 ºC in an incubator shaker (Infors HT Ecotron, Bottmingen, Swizerland) for 24 h at 150 rpm. A 10% inoculum culture of *C. tropicalis* (3.34 ± 0.40 g/L dry cell weight (DCW) corresponding to turbidity of 3.76 ± 0.49 at 600 nm) was used to start subsequent experiments.

3.7.4 Study of Growth Profile of *Candida tropicalis*

Ten percent (v/v) of the adapted *C. tropicalis* inoculum (3.34 ± 0.40 g/L DCW) from a 24 h culture was aseptically added to the hydrolysate growth medium (subsection 3.2.4) for initiating growth profile experiments (Govindaswamy and Vane, 2007 and Sian et al., 2005). The cells were continuously cultivated in 1 L loosely cotton plugged Erlenmeyer flasks containing 250 mL of culture using an incubator shaker at 30 ºC and 150 rpm. The growth of cells was continuously monitored by measuring culture optical density (OD) and dry cell weight (DCW). For growth analysis, 5 mL culture was withdrawn at various time intervals. The OD of culture broth was measured at 600 nm in a quartz cuvette of 1 cm path against a blank consisting of sterile medium with a UV-Vis spectrophotometer (U-1800, Hitachi, Tokyo, Japan). Samples were diluted, with pure medium, to give an OD value less than 2.0 prior to measurement while needed. The initial concentration of inoculum in all experiments was 0.42 ± 0.05 g/L DCW, which corresponds to culture turbidity of 0.60 ± 0.06 at 600 nm. Fresh cell weight (FCW) was measured from 20 h cultured broth by centrifugation at 8000 rpm for 20 min in a refrigerated centrifuge (Eppendorf centrifuge 5810 R, Eppendorf, Germany). The centrifuge tubes (50 mL) were pre-weighed, and after centrifugation, the supernatant was decanted, and the pellet weight was calculated. The DCW was determined by dry weight method (Cronwright et al., 2002 and Govindaswamy and Vane, 2007) as described below.

3.7.5 Dry Cell Weight Measurement

Preparation of Filter Disk

The glass microfibre filter disk (Grade GF/C, 47 mm, Whatman, UK) was placed on an aluminum weighing dish as a support and dried in an oven (Heraeus Instrument, USA) at 105 ºC for 1 h (Cronwright et al., 2002). The heated dish with filter disk was cooled in desiccator, weighed immediately before the experiment and recorded as the initial weight of the dish and filter (W_1). Forceps and gloves were used to handle the aluminum weighing dish.

Analysis

The filter disk was wetted, with a small volume of sterile distilled water, to set it on the filter support. The disk was inserted with wrinkled side up in filtration apparatus by forceps and placed the marked aluminum weighing dish to the side. After assembling filtering apparatus and filter, suction was begun by a vacuum pump. The analysis of DCW was conducted according to the method outlined by Cronwright et al., 2002 and Govindaswamy and Vane, 2007. The cell suspension was mixed well and pipetted 5 mL of it onto the seated glass filter. The filter was washed with three successive double volumes (10 mL) of sterile distilled water to remove the contaminated medium, allowing complete drainage among washing steps, and continued suction for 2 min after the filtration was complete. The filter was carefully removed from filtration apparatus with forceps and transferred to the marked dish. Then the dish was placed in an oven and dried at 105 ºC for 12 h. After the drying, dish was removed from oven, cooled in desiccator for 30 min to balance temperature in a dry atmosphere. Weighed the dish again along with filter disk and recorded as the final weight of the dish and filter disk plus dried cells (W_2). The dry cell weight (DCW) was measured in triplicate by the increment of the filter disk weight after drying, calculated by Eq. (3.17), and was expressed as g/L.

$$\text{Dry cell weight (g/L)} = \frac{W_2 - W_1}{V} \qquad (3.17)$$

Where, W_1 = Weight of dish and filter disk (g); W_2 = Weight of dish and filter disk + dried cell (g) and V = Volume of cell culture employed in the experiment (mL).

3.7.6 Preparation of Crude XR

A 25 mL sample of adapted *C. tropicalis* inoculum was introduced into 1 L loosely plugged Erlenmeyer flask containing 225 mL of hydrolysate growth medium (10% inoculum) and incubated as described before (subsection 3.7.4). Cells were harvested from the culture broth at the end of the exponential growth phase (20 h; OD and DCW values of 11.98 ± 0.89 and 4.87 ± 0.39 g/L, respectively) by centrifugation at

8,000 rpm for 20 min at 4 ºC using a refrigerated centrifuge. The cell pellet was washed twice with ice-cold 0.1 M potassium phosphate buffer of pH 7.0, centrifuged as before and then the pellet was either used directly or stored at –80 ºC until further use. The XR enzyme was isolated from *C. tropicalis* by ultrasonic homogenization as illustrated in Figure 3.6. The washed pellet was resuspended in required volume of ice-cold same buffer to give a fresh cell weight (FCW) to buffer ratio (w/v) of 1:2 (Yokoyama et al., 1995). The cell suspension was submitted to disruption with an ultrasonic cell homogenizer (Omni Ruptor 4000, Omni International, USA) by 20-kHz sonication at pulsing/resting cycles of 1 s for 45 min as outlined by Tomotani et al. (2009). During cell disruption, all operations were conducted on ice. The resulting cell homogenate was then centrifuged at 8,000 rpm for 20 min at 4 ºC to obtain a supernatant solution. For further clarification, the supernatant was centrifuged again at 12,000 rpm for 30 min. The refined supernatant was stored at –80 ºC and used as crude enzyme for assaying xylose reductase (XR) enzyme activity as well as for xylitol synthesis. The XR samples were taken from three disruption procedures carried out independently under the same conditions.

(a) **(b)** **(c)**

Figure 3.6: Preparation of XR from *C. tropicalis* by ultrasonication: (a) cell pellet, (b) homogenization of cells and (c) crude XR solution

3.7.7 Assay of XR Activity

Principle

XR is generally a homodimeric protein and a NADPH-dependent oxidoreductase. XR together with its coenzyme, NADPH, catalyzes the reduction of xylose to xylitol (Figure 3.7). The method employed in XR assay is a spectrophotometric analysis, wherein the oxidation of NADPH to $NADP^+$ is monitored as a decrease in absorbance at 340 nm (Nidetzky et al., 1996, 2003 and Yokoyama et al., 1995). The rate of decrease in A_{340} is directly proportional to the XR enzyme activity in the sample as the enzyme existed at rate limiting concentrations. The unit for XR activity can be expressed in terms of the oxidation of NADPH or reduction of xylose since their molar ratio is 1:1.

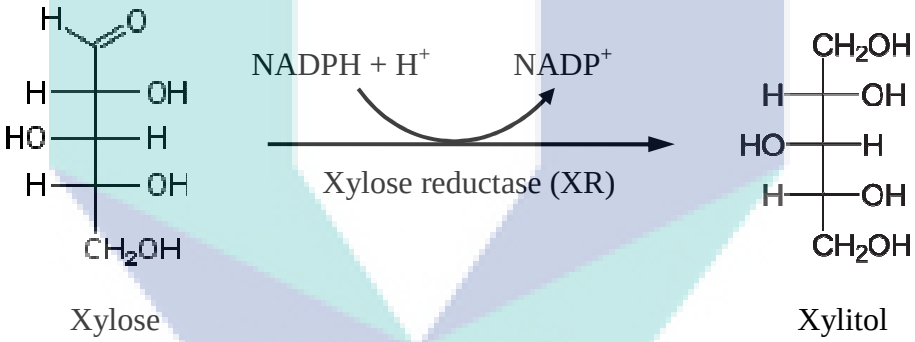


Figure 3.7: Reduction of xylose to xylitol by NADPH-dependent XR

Procedure

The activity of XR was determined spectrophotometrically at 340 nm by monitoring the oxidation of NADPH in a quartz cuvette (1 cm path length) at 25 ºC following the method outlined by Yokoyama et al. (1995). The reaction mixture in the cuvette (3.5 mL) contained 0.2 mL of 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mL of 0.1 M 2-mercaptoethanol, 0.1 mL crude XR, 0.1 mL of 3.4 mM NADPH, and 1.2 mL of sterile ultrapure water. 2-Mercaptoehanol was added in the reaction mixture to inhibit protease activity if present in the crude XR or reaction mixture. The reaction mixture was thoroughly mixed and allowed to stand for 1 min to eliminate the

endogenous oxidation of NADPH to $NADP^+$. The reaction was initiated by adding 0.2 mL of 0.5 M D-xylose. The XR was preboiled in a water bath for 5 min to inactivate the enzyme and added to the control instead of live XR. The rate of NADPH oxidation was measured at 340 nm by UV-Vis spectrophotometer at 1 min intervals for 5 min. The recipe of the buffer and other solutions used in XR activity assay is presented in Appendix A2. One unit (U) of XR is referred to as the amount of enzyme required to catalyze the oxidation of 1 µmol of NADPH per min at pH 7.0 and 25 ºC. Specific enzyme activity was expressed as units of the enzyme per mg of protein (i.e., U/mg).

Calculation of XR Activity

It is known that one molecule of NADPH is consumed per molecule of xylose reduced (Nidetzky et al., 1996). Thus, the oxidation of NADPH (measured by loss of A_{340}) directly correlates with xylose reduction. The rate of decrease in absorbance per min (ΔA_{340}/min) for both samples and controls was determined through Eq. (3.18) while the net rate was calculated by subtracting the control rate from the sample rate using Eq. (3.19). The XR activity in the enzyme sample was calculated using the extinction coefficient of NADPH by the following formulas (Nidetzky et al., 1996 and Zeid et al., 2008) and expressed as U/mL.

$$\Delta A_{340}/\text{min} = \frac{(A_{340}\ \text{at}\ 1\,\text{min}) - (A_{340}\ \text{at}\ 5\,\text{min})}{4\,\text{min}} \quad (3.18)$$

$$\text{Enzyme activity}\,(\text{U/mL}) = \frac{\Delta A_{340}/\text{min}\,(\text{Test}) - \Delta A_{340}/\text{min}\,(\text{Control})}{E^{\text{mM}} \times V_e} \times V_a \times \text{DF} \quad (3.19)$$

Where,

ΔA_{340}/min	=	Rate of decrease in absorbance at 340 nm per min
V_a	=	Total volume of assay (mL)
DF	=	Dilution factor
E^{mM}	=	Millimolar extinction coefficient of NADPH at 340 nm
	=	6.22 mM^{-1} cm^{-1}
V_e	=	Volume of enzyme (mL) added to the assay

To obtain the specific activity, the protein content of the sample was determined and the enzyme activity was divided by the protein value as expressed in Eq. (3.20).

$$\text{Specific activity}\,(\text{U/mg protein}) = \frac{\text{Enzyme activity}\,(\text{U/mL})}{\text{Protein concentration}\,(\text{mg/mL})} \qquad (3.20)$$

3.7.8 Optimum pH and Temperature for XR

The effects of pH and temperature on the activity of XR enzyme were analyzed according to the method of Li et al., 2010 and Sian et al., 2005. The optimum pH for the activity of crude enzyme was determined by the XR assay in the following buffers: 0.1 M sodium acetate buffer (pH 4.0–5.0), 0.1 M potassium phosphate buffer (pH 6.0–8.0) and 0.1 M glycine-NaOH buffer (pH 9.0–10.0) at 25 ºC for 5 min. Optimum temperature of the crude XR was determined by incubating the reaction mixture at various temperatures ranging from 20–80 ºC for 5 min. The XR assay procedure mentioned in subsection 3.7.7 was essentially followed during the study of pH and temperature effects on the enzyme.

3.7.9 Stability of XR

Since XR is an enzyme with a potential technological significance, its stability is of great importance. Thus, the effects of pH, temperature, incubation period, and storage time on the stability of XR were studied as described below:

pH Stability

The pH stability of XR was determined by incubating 0.1 mL of XR with 0.2 mL of different buffers (described in subsection 3.7.8) at different pH at 25 ºC for 1 h without substrate. The standard XR assay method was followed to determine its residual activity at 25 ºC and pH 7.0. The relative activity of the pH treated enzyme was calculated by comparing the activity with that of without pH treatment (Li et al., 2010).

Thermal Stability

The temperature stability of the enzyme was determined by treating 0.1 mL of the enzyme with 0.2 mL of 0.1 M potassium phosphate buffer (pH 7.0) at different temperatures ranging from 20–80 ºC for 1 h and then brought back to 25 ºC. The residual activity was measured by the standard assay method at 25 ºC and pH 7.0. Finally, the relative activity was calculated by comparing the activity with that of without thermal treatment (Li et al., 2010).

Incubation Period Stability

To evaluate the effect of incubation period on the enzyme stability, 0.1 mL of XR was mixed with 0.2 mL of K-phosphate buffer (0.1 M, pH 7.0). The resulting enzyme mix was incubated at 25 ºC without substrate for various incubation periods ranging from 1–48 h before the standard assay to determine XR activity. The residual and relative activities of XR were determined as described above (Li et al., 2010).

Storage Stability

The XR enzyme was prepared on ice in K-phosphate buffer (0.1 M, pH 7.0) according to the procedure described in subsection 3.7.6 and stored at –80 ºC in screw capped vials (2 mL). The remaining activity of XR was monitored with storage time varying from 30–120 days following the standard assay method (subsection 3.7.7). The initial activity of the enzyme was regarded as a standard to calculate the relative activity.

3.7.10 Determination of Enzyme Kinetic Parameters

Principle

Enzyme kinetic parameters were determined to investigate the effect of substrate concentration on the rate of the reaction catalyzed by XR. The reaction rate is defined as

the amount of product generated per unit time, which is expressed by the equation below (Boyacı, 2005 and Mu et al., 2006):

$$\text{Reaction rate } (V) = \frac{\Delta [P]}{\Delta t} \tag{3.21}$$

where ΔP represents the change in the product concentration and Δt is the change in reaction time. The reaction rate depends on the availability of substrate. Unless the substrate is in large excess, the rate decreases with increasing time because the substrate is consumed in the reaction system and its concentration decreases. The characteristics of enzyme-catalyzed reactions are typically interpreted by kinetic parameters such as K_m and V_{max}. These parameters were determined using the Michealis-Menten (M-M) equation and Lineweaver-Burk method (Lineweaver and Burk, 1934). The rate of xylitol production, V (μM/min), versus initial substrate (xylose or NADPH) concentration, [S] (mM or μM), was fitted to the following M-M equation (Eq. (3.22)):

$$V = \frac{V_{max}[S]}{K_m + [S]} \tag{3.22}$$

where V_{max} is a numerical value representing the maximum reaction rate (μM/min) and K_m is the M-M constant (mM). In M-M kinetics, V_{max} value corresponds to the condition where all enzyme molecules are saturated with its substrate. K_m defines the concentration of substrate at which the reaction velocity is half of its maximum value (i.e., $K_m = V_{max}/2$) (Cabezudo et al., 1995). K_m value represents the affinity between enzyme and substrate. A lower K_m value of enzyme indicates a higher affinity for the substrate. The M-M equation was further rearranged to Eq. (3.23), according to which a linear plot between 1/V and 1/[S] was attempted. This double reciprocal relationship is called Lineweaver-Burk plot.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{3.23}$$

The Lineweaver-Burk equation is used to construct a straight line with a slope K_m/V_{max} and an intercept on the y-axis is equal to $1/V_{max}$. The values of intercept and slope were measured by linear regression. This equation corresponds to the equation of a straight line below (Eq. (3.24)):

$$y = mx + b \tag{3.24}$$

where 'm' denotes the linear slope and 'b' is the intercept on the y-axis.

Procedure

The kinetic parameters (K_m and V_{max}) of XR-mediated NADPH-dependent reduction of xylose were determined by measuring initial reaction rate (V). A series of rate measurements were carried out at a fixed and saturating concentration of coenzyme NADPH (300 µM) while the substrate xylose was varied over a range of 40–400 mM, and again, at a constant and saturating concentration of xylose (280 mM) the coenzyme was varied from 15–150 µM according to the method described by Fernandes et al., 2009 and Verduyn et al., 1985a. The reaction mixture had a total volume of 2 mL. Kinetic experiments were conducted in a shaker incubator at defined reaction conditions (as established by response surface study) in 0.1 M K-phosphate buffer (pH 6.5) employing 3% (v/v) (0.33 U/mL of reaction volume) of crude XR. After adding and mixing the last component of the reaction, a 100 µL mixture representing 0 h reaction was withdrawn and boiled in a water bath for 5 min to stop the reaction and stored at –20 ºC until analysis. The remaining assay mixture (1.9 mL) was incubated for 12.25 h at 35 ºC with agitation at 100 rpm. The control contained 60 µL (3%) of a 5 min preboiled XR in place of native XR. At the end of the incubation period, all samples were boiled for 5 min and then frozen at –20 ºC. The reaction samples were analyzed by HPLC for xylitol produced from xylose.

3.7.11 Minimum Inhibitory Concentration of byproducts on XR

The minimum inhibitory concentration (MIC) of selected byproducts in the MWSHH were estimated to observe whether their inhibitory effect on XR activity are significant (i.e., if >15% of inhibition is observed) (Nidetzky et al., 1996) or insignificant. The MIC is referred to as the lowest concentration of a compound required to inhibit the activity of an enzyme *in vitro* over a defined time interval relative to the highest activity (Nickavar and Yousefian, 2011). The MIC values of six undesired products namely glucose, arabinose, acetic acid, furfural, HMF, and LDPs for the isolated XR were determined by a 2-fold serial dilution technique (Isaksson et al., 1991) using a defined reaction medium (0.2 mL of 0.1 M phosphate buffer, 0.2 mL of 0.1 M 2-mercaptoethanol, 0.1 mL of 3.4 mM NADPH, 0.2 mL of 0.5 M pure xylose, and 1.3 mL of sterile ultrapure water) as mentioned in subsection 3.7.7. In serial dilution assay, 2 mL of glucose stock solution (20 g/L) was added to a test tube containing 2 mL of the reaction medium and mixed well. From this test tube, 2 mL was transferred to another tube containing 2 mL of the medium and so on to give final concentrations ranging from 10–0.625 g/L. Finally, 2 mL of the mixed content was discarded from the last test tube to maintain the identical reaction volume (i.e., 2 mL). To the control tube containing 2 mL of the reaction medium, 2 mL buffer instead of inhibitor was added, mixed and then discarded the mixed content (2 mL) as above. After incubation at 25 ºC for 30 min, 0.1 mL of crude XR was added to each test tube and mixed uniformly. All of the test tubes were incubated at 25 ºC and XR activity was determined exactly following the XR assay procedure mentioned in subsection 3.7.7.

The MICs of arabinose, acetic acid, furfural, HMF, and tannic acid were measured by incubating the XR in the absence (as control) or in the presence (as sample) of decreasing order of concentration of byproduct following the same procedure as for glucose with the concentrations ranging from 5–0.313, 8–0.5, 1.5–0.094, 0.2–0.013, and 4–0.25 g/L, respectively. Tannic acid was used as a small lignin degradation model product in this study. The high levels of these compounds were chosen based on the reported maximum values commonly present in different hemicellulosic hydrolysates (Canettieri et al., 2007; Carvalheiro et al., 2004a; Dominguez et al., 1997; Mussatto and Roberto, 2005). To test the synergistic inhibitory effect, required amount of these

byproducts were mixed together in order to give the same concentration of them (4.64 g/L glucose, 2.55 g/L arabinose, 4.14 g/L acetic acid, 0.55 g/L furfural, 0.08 g/L HMF, and 1.55 g/L tannic acid) present in the MWSHH. The MIC of the resulting byproduct's mixture was measured as mentioned above. The MIC experiments were conducted in triplicate and average values ± SD were recorded. The percentage inhibition of XR activity and IC_{50} (inhibitory concentration–50) value were determined through Eq. (3.25) described by Nickavar and Yousefian (2011).

$$I_{XR}\,\% = \frac{(\text{XR activity of control}) - (\text{XR activity of sample})}{\text{XR activity of control}} \times 100 \qquad (3.25)$$

3.8 Enzymatic Xylitol Synthesis

MWS hemicellulosic hydrolysate (MWSHH) was boiled at 100 ºC on a hot plate and its volume was reduced to ½ (half) of the initial volume in order to achieve a 2-fold increase in the xylose content (37.6 g/L). When required, the concentrated MWSHH was diluted with ultrapure water to maintain a targeted xylose concentration. The reaction medium for *in vitro* xylitol synthesis by XR contained 0.1 M potassium phosphate buffer (pH 7.0), crude XR enzyme, and NADPH in 50 mL Erlenmeyer flask as reported by Yokoyama et al. (1995). The reaction was started by the addition of MWSHH as substrate. Preboiled XR was used instead of fresh XR as control. Following thorough mixing of the reaction mixture, a 100 µL volume was withdrawn to use as a zero time reaction, boiled and then stored at –20 ºC until analysis. The remainder of the reaction mixture was incubated at different experimental conditions in an incubator shaker. The assay of the residual XR activity was performed by diluting aliquots taken from the reaction mixture into the respective assay buffer. At the end of desired length of time, the reaction was stopped by heating the reaction mixture in boiling water at 100 ºC for 5 min. The denatured protein in the reaction sample was separated by centrifugation at 8000 rpm for 10 min at 4 ºC. The supernatant was stored at –20 ºC and analyzed for xylitol, xylose, glucose, arabinose, acetic acid, furfural, HMF, and LDPs. All the experiments were performed in triplicate and results presented were the average values with SD.

3.9 OPTIMIZING PROCESS CONDITIONS FOR XYLITOL SYNTHESIS

3.9.1 Parameter Design for Xylitol Synthesis

Parameter design is the primary step in the optimization studies and is employed to select the effective range of each factor. It was implemented with OFAT approach by monitoring the influence of seven test variables such as reaction time, temperature, pH, concentration of xylose, NADPH and enzyme, and agitation rate on the experimental response, xylitol yield. The reaction variables used for each study using OFAT is shown in Table 3.3, which was chosen according to earlier reports on the enzymatic conversion of pure xylose to xylitol (kitpreechavanich et al., 1985; Neuhauser et al., 1997 and Nidetzky et al., 1996). At the beginning of the OFAT experiment on xylitol synthesis, the values of six factors out of seven were kept at a constant level (temperature at 25 ºC, pH at 6.0, xylose concentration at 18.8 g/L, NADPH concentration at 2.83 g/L, enzyme concentration at 5% (v/v) (0.55 U/mL of reaction volume), and agitation rate at 150 rpm) based on the reports (Neuhauser et al., 1997, 1998 and Yokoyama et al., 1995). The first factor was then changed until an optimum value was reached. This optimum value for the first factor was then held constant while the second variable was varied and so on. To verify the OFAT results, the experimental data were analyzed by Minitab® statistical software (version 15, Minitab Inc., USA) and evaluated statistically with the determination coefficient (R^2), correlation coefficient (R), and $Prob > F$.

Table 3.3: Factors and their ranges used in OFAT study for xylitol synthesis

Factors	**Variation of factors**						
	Time (h)	**Temp (ºC)**	**pH**	**Xylose (g/L)**	**NADPH (g/L)**	**Enzyme (%, v/v)**	**Agitation (rpm)**
Time	2–18	25	6.0	18.8	2.83	5	150
Tempera-ture	10	20–70	6.0	18.8	2.83	5	150
pH	10	30	4.0–9.0	18.8	2.83	5	150
Xylose conc.	10	30	7.0	9.4–37.6	2.83	5	150
NADPH conc.	10	30	7.0	18.8	1.17–5.32	5	150
Enzyme conc.	10	30	7.0	18.8	3.66	2–6	150
Agitation rate	10	30	7.0	18.8	3.66	3	50–150

3.9.2 Identifying the Significant Variables using FFD

In the second step of optimization process, fractional factorial design (FFD) was employed to screen the factors significantly affecting xylitol production from hemicellulosic hydrolysate. FFD is very efficient, economical, and widely used experimental tool for screening many factors to find the significant ones, and for determining the main and interaction effects (Montgomery, 2001). The statistical software Design Expert® was applied for the experimental design and analysis of the observed data throughout the screening process. Five independent variables considered for the factorial design were reaction time (X_1), temperature (X_2), pH (X_3), NADPH concentration (X_4), and enzyme concentration (X_5). Each variable was examined at a high (coded +1) and low (coded -1) level. The center points were the runs with the basal level conditions (coded 0). According to the results of OFAT study, other two factors xylose concentration and agitation rate were kept constant at 18.8 g/L and 100 rpm, respectively, in the subsequent experiments. The high level of each factor was set far enough from the low level to identify the process factors having significant influence on the production of xylitol. The critical ranges of the selected input variables were determined by preliminary single factor experiment. Table 3.4 shows the independent variables for screening process using FFD. A 2^{5-1} fractional factorial design (i.e., ½ fraction of the 2^5 full factorial design) was applied to analyze the statistical significance of each parameter affecting xylitol synthesis and consequently this design included 16 combinations plus 6 replicates at the center point leading to 22 sets of experimental runs. Xylitol yield ($Y_{p/s}$) was taken as the response or output variable of the factorial design experiments. Each run was carried out in triplicate in random order to avoid systematic errors, and the mean values with SD were recorded. The experimental design and results were analyzed through a first order polynomial equation (Eq. (3.26)) based on the method of Montgomery (Montgomery, 2001).

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i \tag{3.26}$$

where Y is the value of the response variable; b_0 is the interception coefficient; b_i represents the coefficients of the linear parameters; X_i represents the coded independent

variable and *k* is the number of variables. The statistical significance of the regression coefficients was measured by *F-test* value and the model terms were selected or rejected based on the value of *F*-ratio (*Prob* > *F*) or significance. The statistical analysis of the first degree model equation was conducted via ANOVA. This analysis comprised of *F-test*, its associated probability, correlation coefficient (*R*) and determination coefficient (R^2), which measures the proportion of variance explained by the created model.

Table 3.4: Independent variables for screening process using FFD

Factors	Unit	Symbol	Range and levels		
			-1	0	+1
Reaction time	h	X_1	8	10	12
Temperature	ºC	X_2	25	30	35
pH		X_3	5.0	6.0	7.0
NADPH conc.	g/L	X_4	2.0	2.83	3.66
Enzyme conc.	% (v/v)	X_5	2	3	4

3.9.3 Optimization of Critical Variables for Xylitol Synthesis

After screening the variables by FFD, the 3 most important input variables namely reaction time, temperature and pH were selected for further evaluation of their impacts on xylitol production using CCD in RSM. The CCD was adopted to illustrate the nature of response surface in the experimental region and elucidate the optimum combinations of the factors involved. Ranges and levels of variables tested in CCD for xylitol synthesis are shown in Table 3.5. The selected variables were examined at five different levels (relatively low, low, basal, high, relatively high) coded (-2, -1, 0, +1, +2) (Table 3.5). The other 4 variables such as xylose concentration, agitation rate, NADPH concentration (X_4), and enzyme concentration (X_5) in all CCD experiments were set at their middle levels as 18.8 g/L, 100 rpm, 2.83 g/L, 3% (v/v) (0.33 U/mL), respectively, based on the results of both the OFAT and FFD studies. According to the full factorial 2^3 CCD, a total of 20 experiments including eight factorial points, six axial points ($\alpha = \pm 2$) and six repetitions at the center point were executed in a single block. This design was applied for optimizing critical variables when xylitol yield ($Y_{p/s}$) and volumetric productivity (Q_P) were taken as dependent variables or responses. The experimental runs were conducted in random order. To predict the optimum point,

experimental results were adjusted to Eq. (3.27), a second order polynomial model equation, as outlined by Montgomery (Montgomery, 2001).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (3.27)$$

where Y presents the predicted value of the response variable; X_1, X_2, and X_3 are the coded independent variables corresponding to reaction time, temperature and pH, respectively; b_0 is the regression coefficient at the center point; b_1, b_2, and b_3 represent the linear term coefficients; b_{11}, b_{22}, and b_{33} represent the quadratic term coefficients; b_{12}, b_{13}, and b_{23} are the interaction term coefficients. The developed second order polynomial model was statistically evaluated by analyzing the values of regression coefficients, ANOVA, *F*- and *P*-values. The quality of fit of the regression model was expressed through the coefficient of determination (R^2) and correlation (R). The statistical software (Design Expert® 6.0.8) was used to design CCD experiment as well as to construct a quadratic regression model in order to predict the optimum conditions considering linear, quadratic, and interaction effects on xylitol yield and productivity and to plot response surface. For maximum xylitol synthesis, an optimum setting of the variables level was obtained by numerical analysis depending on the desirability criterion. Five sets of experiments were performed at model recommended optimal conditions to validate the CCD model developed. A final experiment was also performed in triplicate, to confirm the model. To compare the process responses, a control reaction was conducted using commercial pure xylose under the optimum conditions established by CCD.

Table 3.5: Ranges and levels of variables tested in CCD for xylitol synthesis

Input variables	Symbol	Range and levels				
		-α	-1	0	+1	+α
Reaction time (h)	X_1	10	11	12	13	14
Temperature (ºC)	X_2	31	33	35	37	39
pH	X_3	5.5	6.0	6.5	7.0	7.5

α = ± 2

3.10 ANALYTICAL METHODS

3.10.1 Estimation of Xylitol, Sugars, Furfural and HMF by HPLC

High performance liquid chromatography (HPLC) was used to estimate xylitol, sugars, furfural, hydroxymethylfurfural (HMF), and acetic acid concentration. This analysis was carried out using an Agilent 1200 chromatograph (Agilent, USA) equipped with a refractive index detector (RID) and an ultraviolet diode array detector (UV-DAD). The concentrations of xylitol, xylose, glucose, arabinose, and acetic acid were measured by HPLC with a RID and a Rezex RHM Monosaccharide H^+ column (300 mm × 7.8 mm; Phenomenex, USA) in combination with a guard column (50 mm × 7.8 mm; Phenomenex, USA) operated at 80 ºC. Ultrapure water was used as mobile phase at a flow rate of 0.6 mL/min and 20 µL of filtered sample was injected by auto sampler. The mobile phase was previously vacuum filtered using 0.45 µm nylon membrane filter (Membrane solutions) and degassed in an ultrasonic bath (Daihan, Korea) for 60 min to remove any dissolved air. Furfural and HMF concentrations were also measured by HPLC but with an UV-DAD set at 276 nm and a Zorbax eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 µm; Agilent, USA) at 25 ºC. In this case, the mobile phase was acetonitrile/water (1:8) with 1% (v/v) glacial acetic acid at a flow rate of 0.8 mL/min with an injected sample volume of 20 µL. This mobile phase was also vacuum filtered and degassed as previous one. Standard solutions were prepared in ultrapure water in the range of 0.1–8 g/L and the HPLC system was calibrated with xylitol, D-xylose, D-glucose, L-arabinose, acetic acid, furfural, and HMF standards. The samples of hemicellulosic hydrolysate and xylitol solution were diluted with ultrapure water (1/5, v/v). All the diluted samples and standard solutions were filtered with NY 0.45 µm syringe filter (Membrane solutions) into HPLC vials before running analysis. The above mentioned compounds exist in the sample were identified and quantified by comparing their retention times to that of the respective standards.

3.10.2 Estimation of LDPs by Prussian Blue Method

Principle

The total concentrations of lignin degradation products (LDPs) in the hydrolysate were determined spectrophotometrically according to the modified Prussian blue method (Graham, 1992) using tannic acid as standard. The phenolic substances react with $K_3Fe(CN)_6$ and $FeCl_3$ in 0.1 N HCl in the presence of an excess of Fe^{3+} to develop a blue color whose density is proportional to the amount of polyphenols present. This redox reaction, known as the Prussian blue method, is simple, rapid, economical, and is used universally for the estimation of total phenolics. The procedure is detailed below:

Procedure

The reagents used in the Prussian blue method were prepared in the laboratory and are given in Appendix A3. To determine the total LDPs, the hemicellulosic hydrolysates were prepared by diluting 100 times (v/v) with ultrapure water in test tubes. Three mL of diluted sample was dispensed in duplicate sets of test tubes (20 mL). To each test tube, 1 mL of 0.016 M $K_3Fe(CN)_6$ solution was added followed immediately by the addition of 1 mL of 0.02 M $FeCl_3$ in 0.1 N HCl. The contents of the tubes were mixed thoroughly by vortex (Classic vortex mixer, VELP scientifica, Italy) and incubated at 25 ºC for 15 min. Then, 3 mL of 6.03 M H_3PO_4 was added, contents were mixed and left at 25 ºC for 2 min. Finally, 2 mL of 1% gum arabic was added and mixed vigorously. The color intensity was measured at 700 nm with 1 cm quartz cuvette against a reagent blank consisting of all of the reagents except phenolic compound by using UV-Vis spectrophotometer.

Construction of Standard Curve

A 50 µg/mL stock solution of tannic acid was prepared by dissolving it in ultrapure water. A series of standard solutions was prepared from the stock solution by sequential dilution with ultrapure water, the concentrations of tannic acid being 0.83,

1.66, 3.32, 6.64 and 13.28 µg/mL. Three mL of each solution was taken in individual test tube in duplicate. Color development was done as described under the procedure and the absorbance was measured at 700 nm. The tube containing 3 mL of ultrapure water instead of tannic acid was considered as reagent blank. All the measurements were done in triplicate and a standard curve was constructed by plotting the amount of tannic acid added against their respective absorbance as shown in Figure 3.8. The amount of LDPs was determined from the standard curve based on tannic acid.

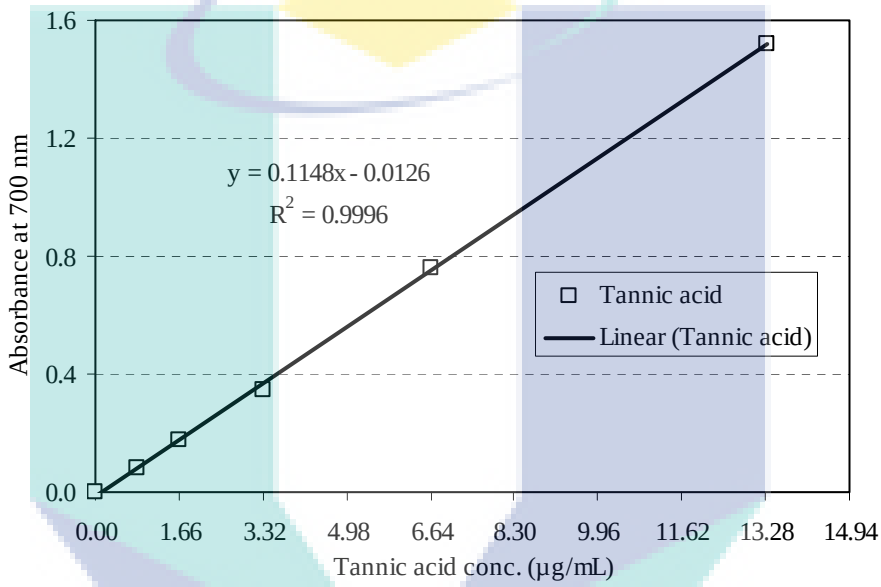


Figure 3.8: Standard curve of tannic acid for the determination of LDPs

3.10.3 Determination of Protein by Lowry Method

Principle

The total protein in the enzyme samples was determined following the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. The basic principle of this method is the reactivity of the peptide nitrogen(s) with copper (II) ions under alkaline condition and the subsequent reduction of the yellow colored Folin-Ciocalteu reagent (FCR; phosphomolybdic phosphotungstic acid) to heteropolymolybdenum blue through the copper-catalyzed oxidation of aromatic amino acids. Three individual determinations on each crude enzyme sample were averaged in order to calculate the specific activity of XR.

Procedure

Samples were prepared by diluting 10 µL of crude XR with ultrapure water in test tubes to a total volume of 250 µL (i.e., 25-times dilution). The reagents for Lowry assay were freshly prepared and are presented in Appendix A4. To each tube 2.5 mL of Lowry reagent (Reagent C) was added and mixed well. The reaction mixture was incubated at ambient temperature. After 10 min, 250 µL of FCR was added to each tube, vortexed and incubated again for 30 min. Optical density was recorded at 750 nm using UV-Vis spectrophotometer in a quartz cuvette (1 cm) against a reagent blank.

Preparation of Standard Curve

A series of BSA standards (0, 25, 50, 100, 150, 200, and 250 µL/tube) was prepared from the stock solution (1 mg/mL) and made a final volume of 250 µL with ultrapure water (the final concentrations of BSA being 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). The test tube containing 250 µL of ultrapure water instead of BSA with all other reagents was taken as reagent blank. The BSA standard solution was treated as described above in the procedure and the intensity of the resulting blue color was detected at 750 nm. All the determinations were carried out in triplicate and a calibration curve for BSA was built by plotting the absorbance versus the respective amount of BSA as shown in Figure 3.9. The protein contents in the samples were calculated from the standard curve and expressed in mg/mL.

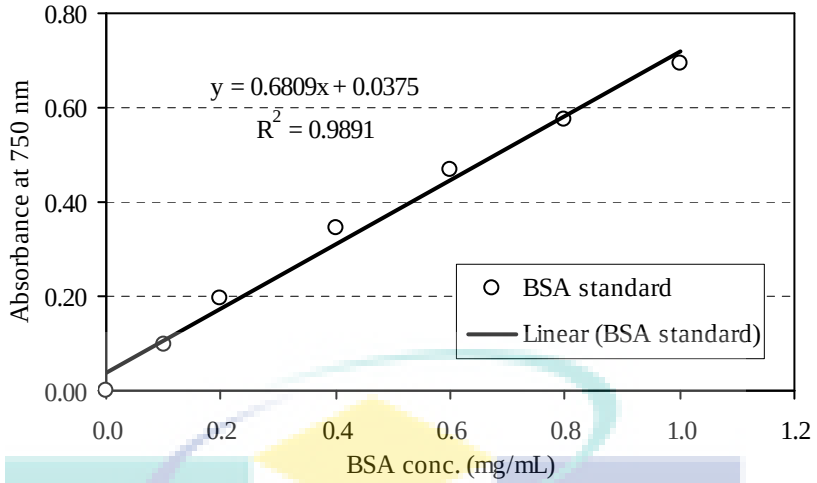


Figure 3.9: Standard curve of BSA for the determination of protein

3.11 CONCLUSION

In any research, methodology is a vital organ. It must be appropriate, modern and efficient. A methodology must be elaborated in a clear and understandable manner to enable a reader to reproduce the work when necessary. In this chapter, the experimental works have been elaborated in a simple and understandable manner. The process optimization strategies and the analytical techniques have been addressed in this chapter. At the beginning of the chapter, the experimental methods for MWS characterization, hydrolysis of MWS, parameter design, kinetic modeling of MWS hydrolysis, and optimization of xylose recovery have been described. Then, XR preparation and characterization have been outlined. Finally, optimization approaches (parameter design, screening, and optimization) for xylitol synthesis have been presented. The kinetics of xylose reduction and analytical methods has also been presented.

CHAPTER 4

CHARACTERIZATION OF MATERIALS

The present study was carried out to synthesize xylitol from xylose of MWS biomass by enzymatic bioconversion, which includes MWS characterization, xylose recovery, XR production and characterization, and xylitol bioproduction. Results and discussion of this work are embodied in three chapters numbered as chapter 4: characterization of materials, chapter 5: recovery of xylose from MWS, and chapter 6: synthesis of xylitol from MWS hemicellulosic hydrolysate (MWSHH). In this chapter the experimental results of MWS analyses and XR characterization including growth profile of *C. tropicalis*, XR activity, stability and enzyme kinetic parameters are presented. The characterization of MWS biomass and XR enzyme is crucial in the determination of the theoretical yield of xylose from the biomass and that of xylitol from xylose by XR, respectively. A detailed discussion on both aspects has also been made in this chapter.

4.1 INTRODUCTION

The raw materials used in the present study were *Meranti* wood sawdust (MWS) and xylose reductase (XR). MWS is a lignocellulosic waste of sawmill. Lignocellulosic biomasses are attractive raw materials for manufacturing a variety of specialty chemicals including xylitol and biofuels due to their extensive distribution, renewability, and availability in huge quantities at very low cost. Lignocellulose, the major building block of all plant biomasses, is composed of cellulose, hemicellulose, lignin, and smaller contents of extractives, ash, pectins, and proteins. The percent composition of these components can vary with growth conditions, age and plant

species (Kumar et al., 2009 and Pérez et al., 2002). In addition, the composition of different constituents varies according to the biomass type such as hardwoods, softwoods and grasses (Kumar et al., 2009 and Prakasham et al., 2009). It was reported that hardwoods generally contain a high amount of hemicellulose but low lignin content than softwoods (Kumar et al., 2009; Palmqvist and Hahn-Hägerdal, 2000b and Thomas et al., 2011). In lignocellulosic materials (LCMs), cellulose, hemicellulose and lignin are closely associated with each other and build a complex architecture. The presence of lignin in the LCMs is one of the principal drawbacks of their use in bioprocess because it makes lignocellulose resistant to degradation by chemical and biological agents (Balat et al., 2008; Prakasham et al., 2009 and Taherzadeh and Karimi, 2008). A wide variety of lignocellulosic biomasses such as corncobs (Dominguez et al., 1997), brewery's spent grain (Mussatto and Roberto, 2005), *Eucalyptus* wood (Villarreal et al., 2006), palm oil empty fruit bunch fiber (Rahman et al., 2007), rice straw (Liaw et al., 2008), soyabean hull (Michel et al., 2008), olive tree pruning (Romero et al., 2010), sugarcane bagasse (Prakash et al., 2011 and Rao et al., 2006), and sweet sorghum bagasse (Liu et al., 2012) have been evaluated as source of raw materials for xylose production. The use of wood sawdust as a renewable bioresource to produce value-added products has not yet been reported. For this research work, MWS was selected as the raw material because of its availability, renewability and abundant in the wood processing industries. It is important to characterize MWS biomass for the determination of its main structural constituents.

XR is an oxidoreductase enzyme normally present in the cytoplasm of yeasts and filamentous fungi (Woodyer et al., 2005 and Zhao et al., 2009). It is a member of the aldose reductase family of enzymes on the basis of sequence and structural similarities (Woodyer et al., 2005). This enzyme has potential applications in the biotechnological production of xylitol, sorbitol, and ethanol from xylose (Rawat and Rao, 1996 and Zhao et al., 2009), which make the enzyme a focus of interest. The utilization of high priced commercial xylose limits the large-scale production of XR as well as its industrial application for manufacturing xylitol and other bioproducts. This issue has encouraged the author to work toward the development of improved techniques to lower the costs of XR preparation. Thereby, the use of xylose-rich MWS hemicellulosic hydrolysate (MWSHH) for XR preparation from yeast strains can be

interesting from an economic point of view. XR is not commercially available despite a large number of reports found in the literature on the important use of this enzyme, as well as a description of downstream processing to separate it from yeasts (Rawat and Rao, 1996; Tomotani et al., 2009; Yokoyama et al., 1995 and Zhao et al., 2009).

Xylose-fermenting yeast under the genus *Candida* is still regarded as the best source of XR among the microorganisms. As a result, XR from *Candida* yeast has been isolated and characterized in the last few decades (Lee et al., 2003; Mayr et al., 2000; Nidetzky et al., 2003; Tamburini et al., 2010; Verduyn et al., 1985a; Wang et al., 2007 and Yokoyama et al., 1995). XR normally prefers NADPH as coenzyme (Yokoyama et al., 1995), but in some cases it utilizes both NADH and NADPH (Mayr et al., 2000; Neuhauser et al., 1997 and Verduyn et al., 1985a). The variation in coenzyme specificities is proposed to regulate the redox potential balance among nicotinamide coenzymes under different growth conditions of yeasts (Hahn-Hägerdal et al., 2001; Nidetzky et al., 2003 and Woodyer et al., 2005). The functional enzymes from *P. stipitis* (Rizzi et al., 1988), *N. crassa* (Rawat and Rao, 1996), and *C. tropicalis* (Yokoyama et al., 1995) consist of two identical subunits. In this study, XR was isolated from yeast *Candida tropicalis* IFO 0618 because it is one of the most efficient xylitol producers (Horitsu et al. 1992 and Granström et al., 2002a).

It is necessary to isolate and characterize XR from the potential xylose-fermenting yeast in order to explore its maximum efficiency on xylose to xylitol bioconversion. The application of XR may offer an economic interest over the chemical and microbial reduction of xylose to xylitol. Therefore, easily obtainable and highly active XR is desirable for *in vitro* enzyme-based production of xylitol. Although XR from different yeasts was characterized, the enzyme has not yet been isolated and studied from yeast grown in lignocellulosic hydrolysate-based medium. The aim of this study was to characterize the raw materials such as MWS and XR, which are used in the subsequent experiments.

4.2 RESULTS AND DISCUSSION

4.2.1 Characterization of *Meranti* Wood Sawdust

Generally, wood sawdust is used as fuel in manufacturing industries and in local utilities with a relatively low heating value (Arends and Donkersloot-Shouq, 1985 and Harkin, 1969). The application of sawdust as raw material to produce high value products could be a good alternative to manage this abundant waste. A detailed knowledge of the MWS biomass composition is necessary for calculating the theoretical yield in polysaccharide as well as for assessing the reactivity of constituents present in the biomass. The analyses of MWS were done in order to determine the principal structural components using standard methods. The experimental results achieved from the chemical analyses of MWS are briefly discussed below.

Composition of MWS

The raw material used in this experiment was oven dried (at 105 ºC for 12 h) MWS biomass with a particle size smaller than 0.5 mm. The main chemical composition of MWS is shown in Table 4.1. The composition of MWS was determined according to the standard methods (as outlined in subsection 3.5). The MWS contains cellulose, hemicellulose and lignin as the major biopolymers with a value of 41.06, 33.20, and 22.23%, respectively (Table 4.1). It is also estimated that this biomass is comprised of more than 29% xylan, a sugar polymer made of xylose. The major polymeric components of MWS were in the range of those of other wood materials stated in the literature (Balat et al., 2008 and Sinağ et al., 2009). The xylan content of the MWS (29.22%) fell within the range 11–35% that has been reported for hardwoods and agricultural residues (Nigam and Singh, 1995 and Parajó et al., 1998b). The high amount of xylan in MWS rendered this biomass adequate for xylose production. It was suggested that lignocellulosic biomasses with a content of over 20–35% xylan can be utilized for the industrial production of xylose or furfural (Kim and Dale, 2004; UNCTAD/GATT, 1979; Vázquez et al., 2007). If xylan polymer is assumed to be completely converted to xylose without further degradation, then the initial

concentration of xylan [Xn_p] expressed as potential concentration (PC) of xylose is calculated through Eq. (4.1) outlined by Vázquez et al. (2007).

$$[Xn_p] = \frac{150}{132} \times \frac{CXn_0}{\text{LSR}} \times 5 = 20.75 \text{ g xylose/L} \quad (4.1)$$

where CXn_0 is the initial content of xylan in the MWS (29.22 g xylan/100 g MWS on o.d.b.), LSR is the liquid/solid ratio (8 g liquid/g of MWS), and 150/132 is the stoichiometric factor giving the interrelationship between xylose and xylan. The PC of xylose is defined as the quantitative conversion of polymer xylan to xylose. The PC of xylose (X_p) released in the hemicellulosic hydrolysate was calculated to be 20.75 g/L.

Table 4.1: Main constituents of *Meranti* wood sawdust (oven dry basis)

Constituents	Content (%, w/w)
Cellulose (alpha)	41.06 ± 0.03
Hemicellulose	33.20 ± 0.07
Xylan (a component of hemicellulose)	29.22 ± 0.08
Lignin (acid insoluble)	22.23 ± 0.05
Extractives	3.08 ± 0.05
Ash	0.43 ± 0.04

4.2.2 Characterization of Xylose Reductase

Xylose derived from MWS can be used as an alternative carbon source for yeast cultivation as well as for the production of XR. XR is one of the key enzymes for the production of xylitol, thus understanding the mechanisms that regulate its activity could help in establishing optimum conditions for xylitol synthesis. The interest in XR preparation from xylose-assimilating organisms could be enhanced if the needed xylose can be obtained from the cheap LCMs. The purpose of this experiment was to isolate XR from *C. tropicalis* grown in MWS hydrolysate-based medium and to characterize it. The determination of XR functional properties is necessary to understand its relative roles in the bioconversion of xylose to xylitol. The results of XR characterization along with discussion are addressed in the following subsections.

Growth Profile of C. tropicalis

In order to obtain cells from the exponential growth phase, it is required to construct a typical growth curve of *C. tropicalis*. A culture should be harvested during its exponential phase because it has been observed that yeast cells contain the highest protein content at this growth phase (Sampaio et al., 2009 and Webb and Lee, 1992). For determining growth curve of adapted *C. tropicalis* in terms of optical density (OD) and dry cell weight (DCW) as a function of time, cells were cultivated continuously at 30 ºC and 150 rpm. Figure 4.1 presents the growth profile of adapted *C. tropicalis* cultivated on MWS hydrolysate growth medium. The curves for OD and DCW were of the same pattern as obvious from the Figure 4.1. The results also demonstrated that the growth curve of the adapted yeast strain in the MWS hydrolysate growth medium showed the standard pattern of lag, log, stationary, and death phases. The experimental data of the growth profile of *C. tropicalis* in hydrolysate medium are shown in Appendix B (Table B.1).

The lag phase refers to the time of inoculation of the yeast strain in the culture medium with a negligible increase in cell density. During this time, *C. tropicalis* cells were adapted with their new environment (such as pH, temperature, nutrients, etc.). It was observed that a period of 2 h was required for acclimation of the strains with an initial OD and DCW values of 0.60 and 0.42 g/L, respectively, and further cultivation led to an exponential growth phase (Figure 4.1). Cells became highly active after 2 h, and they fully utilized the available nutrients. This phase is called exponential or log phase because during this period of time the strains were growing and dividing at a maximum possible rate provided by their genetic potential, the nature of the medium and the cultivation conditions. During this phase the cells were divided and doubled in number at regular intervals. At this phase, the yeast population demonstrates uniform chemical and physiological properties (Govindaswamy and Vane, 2007 and Ko et al., 2008). The exponential phase lasted between 3 and 20 h, and the values of OD and DCW were found to be 11.98 and 4.87 g/L, respectively, at 20 h of incubation.

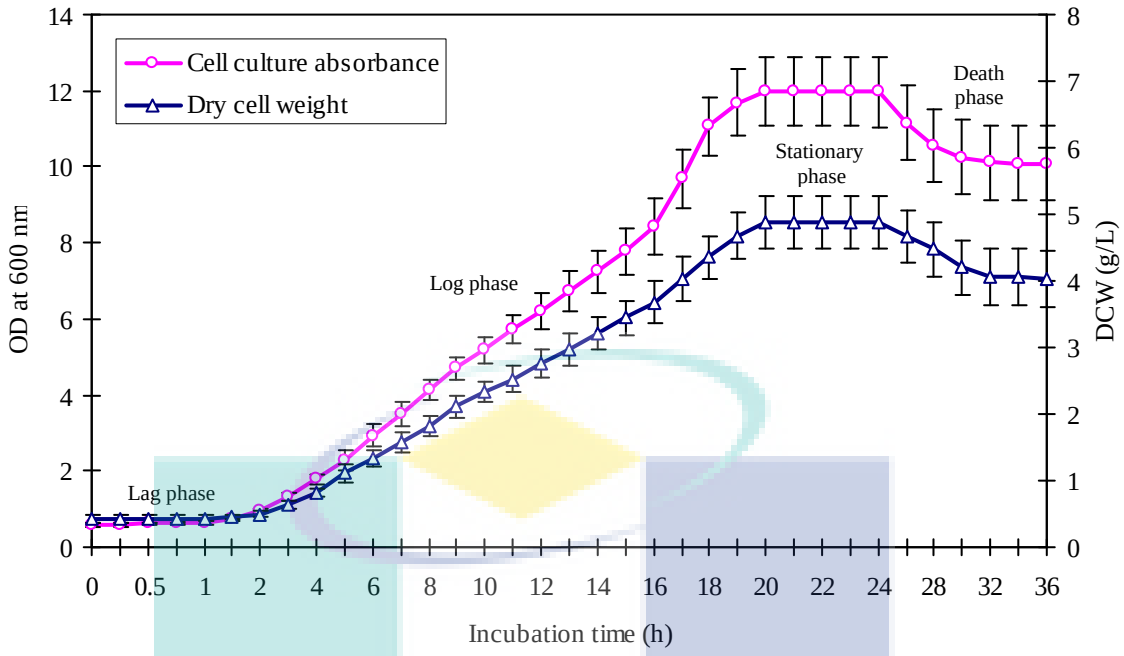


Figure 4.1: Growth profile of adapted *C. tropicalis* in terms of OD and DCW cultured on MWSHH-based medium. Data points and error bars represent the mean values and standard deviations, respectively, from 3 separate experiments

The exponential period is followed by the stationary phase during which the values of OD and DCW were fixed, and the growth curves become horizontal. In this phase, the total number of viable cells remained static. This resulted from a balance between cell division and death or simply due to cessation of proliferation though remaining metabolically active. Microbial strains enter the stationary phase due to several reasons. One of the factors is nutrient limitation. When the availability of the nutrient is severely depleted, cell growth would be ceased. Cell growth is also ceased due to the accumulation of toxic waste and secondary metabolic products (García et al., 1997). The stationary phase was observed from 21–24 h with the constant values of OD and DCW (11.99 and 4.88 g/L, respectively). Death phase is the last phase of microbial growth profile. This phase began after 24 h and the OD and DCW values reduced with incubation time. During this phase, the overall number of cells decreases so the death rate is greater than the birth rate. The detrimental environmental changes such as nutrient deprivation and the build-up of toxic compounds lead to the decline in number of viable cell, which are characteristics of the death phase. The study on growth profile was carried out to see if there is any change in their growth pattern during adaptation of the yeast strain to lignocellulosic hydrolysate-based medium.

The correlation between DCW and OD at 600 nm was elucidated as shown in Figure 4.2. A plot of DCW (g/L) versus OD_{600} gave a straight line up to an optical density of 8.44 (OD reading were taken after diluting the sample while needed), which corresponds to 3.68 g/L yeast biomass with a linear regression (R^2) value of 0.9992 (Figure 4.2). The relationship among DCW and OD is given by Eq. (4.2) as reported by Govindaswamy and Vane (Govindaswamy and Vane, 2007). It is noted that an OD_{600} value of 1 resulted 0.55 g/L DCW (i.e., 1 OD_{600nm} = 0.55 g/L DCW) after linear correlation between absorbance and DCW.

$$\text{Dry cell weight (g/L)} = 0.421(OD_{600}) + 0.132 \quad (4.2)$$

The growth profile study clearly indicated that *C. tropicalis* cells were able to grow on medium containing crude MWS hydrolysate as the carbon source. Therefore, MWS hydrolysate-based medium was used as the cell biomass production medium.

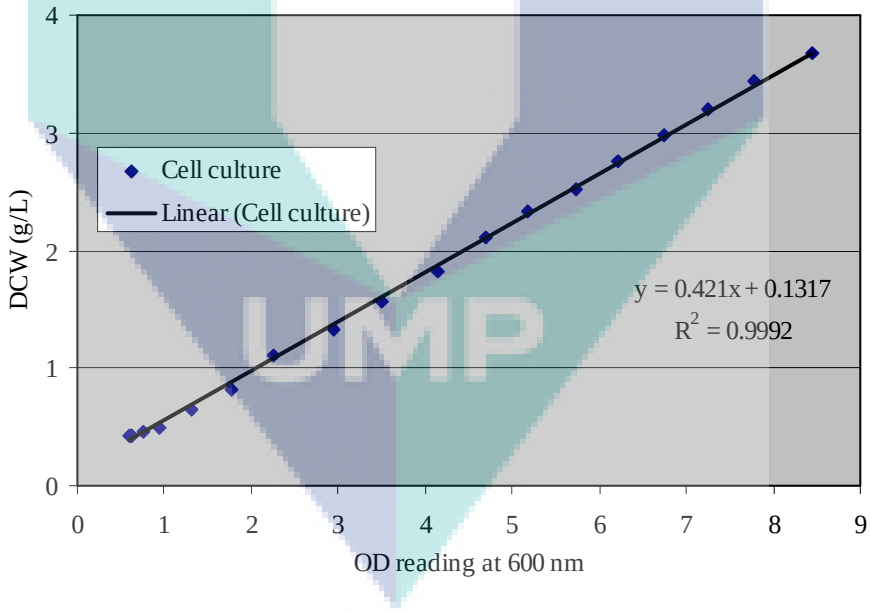


Figure 4.2: Correlation curve of yeast culture OD at 600 nm and its DCW

XR Activity Assay

Xylose reductase (XR), an intracellular enzyme, is an oxidoreductase that mediates the reduction of xylose to xylitol with the concomitant oxidation of NAD(P)H to $NAD(P)^+$. XR was isolated from adapted *C. tropicalis* cells and found that it was

completely specific to NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) coenzyme for its activity. It was also found that the activity of XR for NADH was not detectable. These outcomes are in agreement with the previous reports that the NADPH-dependent XRs exhibited no activity with NADH in *C. tropicalis* (Yokoyama et al., 1995), *C. utilis* and certain strains of *Candida tenuis* (Bruinenberg et al., 1983). In this study, XR activity was determined spectrophotometrically by measuring the amount of NADPH oxidized after a timed reaction coupled with the reduction of xylose to xylitol at 25 ºC and pH 7.0. The volumetric activity of NADPH-dependent crude XR determined was 11.16 U/mL with the corresponding specific activity of 0.91 U/mg of protein. The concentration of protein in the crude XR solution was 12.22 mg/mL. The activity and coenzyme specificity of XR from various yeast strains have been studied (Granström et al., 2002b; Verduyn et al., 1985a and Zeid et al., 2008). It was reported that XR in the xylitol-producing yeasts (such as *Candida intermediae*, *C. parapsilosis*, *C. silvanorum*, *C. tropicalis*, *Kluyveromyces fragilis*, *K. marxianus*, and *Torulopsis molishiama*) is specific for NADPH; and in the ethanol-producing yeasts (such as *P. stipitis*, *C. shehatae*, and *Pachysolen tannophilus*) is specific for both NADPH and NADH. Nidetzky et al. (2003) reported that the xylose-fermenting yeast *C. intermedia* generates two isoforms of XR, one is NADPH-dependent (i.e., monospecific XR; msXR), and another preferred NADH about 4-folds over NADPH (i.e., dual specific XR; dsXR).

Analyses of XR Stability

It is crucial to pay much attention on the maintenance of biocatalytic function of XR including its activity and stability. Thorough understanding of the enzyme inactivation factors will enable one to improve the enzyme activity and stability. The results obtained from the analyses of XR activity and stability based on the effect of pH, temperature, incubation period, and storage time, are described below.

Effect of pH on XR Activity and Stability

To determine the optimum pH, XR activities were monitored following the standard assay procedure within the pH range from 4.0–10.0 at 25 ºC. The optimum pH and pH stability are shown in Figures 4.3 and 4.4, respectively. The adapted *Candida*

tropicalis IFO 0618 XR exhibited optimum activity against xylose at pH 7.0 (Figure 4.3) that is in accordance with some XRs isolated from *Pachysolen tannophilus* (Ditzelmüller et al., 1984), *C. intermedia* (Nidetzky et al., 2003) and *C. parapsilosis* (Lee et al., 2003). It was observed that XR exhibited negligible activity at pH 4.0 (6.12% of the original activity) and 9.0 (only 4.34%). This result reflected that the XR needs a near-neutral pH range to catalyze its reactions, and a higher pH was not favorable for the enzyme to conduct reduction-oxidation (redox) activities. It is reported that most of the XRs showed optimum pH ranging from 5.0–7.0 (Ditzelmüller et al., 1984; Morimoto et al., 1987 and Ronzon et al., 2012). At extreme pH (>7.0) condition, XR activity gradually declined and at pH 8.0 the activity was 24.11% of the original value (11.16 U/mL) (Figure 4.3). The reduction in activity is assumed to be due to the limited ionization of groups involved in substrate binding and catalysis or in enzyme-coenzyme complex formation (Nidetzky et al., 2003). Moreover, extreme pH may cause partial denaturation of the enzyme. The enzyme completely lost its activity at pH 10.0 (100% of original activity) perhaps due to the enzyme inactivation. The activity, 11.16 U/mL, obtained at 25 ºC and pH 7.0 was taken as 100%.

In order to test the influence of pH on the stability of XR, the enzyme activity was measured after incubation at different pH (pH 4.0–10.0) for a period of 1 h. The stability of enzyme affected by pH value is important as it leads the enzyme to undergo reversible or irreversible structural changes under severe pH conditions (Li et al., 2010). The crude XR remained stable within pH 6.0–7.0 at 25 ºC and lost most of the activity when incubated at pH 4.0 and 8.0, retaining only 3.1 and 8.32%, respectively (Figure 4.4). It was also found that XR became fully inactive while incubated at pH 9.0–10.0.

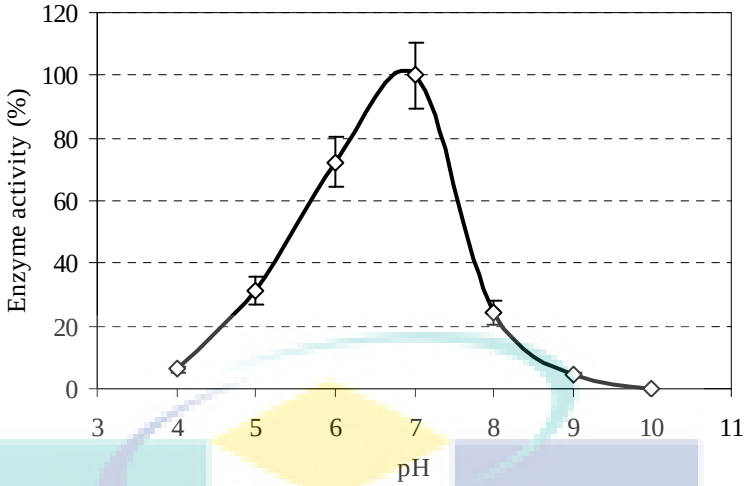


Figure 4.3: Optimum pH for XR activity

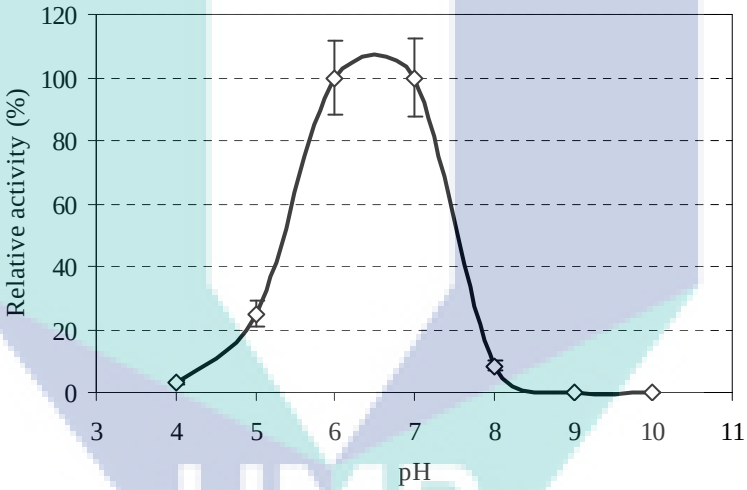


Figure 4.4: pH stability of XR

Effect of Temperature on XR Activity and Stability

The optimum temperature was calculated by assessing XR activities at various temperature range of 20–80 ºC at pH 7.0. The results of XR activity at different temperatures are illustrated in Figure 4.5. It was observed that the optimum temperature for *C. tropicalis* XR was 25 ºC with xylose as substrate (Figure 4.5). A number of studies have been performed by several researchers on XR from *Candida tenuis* (Neuhauser et al, 1998), *C. intermedia* (Mayr et al, 2000) and *C. guilliermondii* (Rodrigues et al, 2006) also discovered 25 ºC as the suitable temperature, similar with XR isolated from adapted *C. tropicalis*. The XR from *P. stipitis* possessed a higher

optimum temperature at 38 ºC (Verduyn et al., 1985a). The effect of temperature on XR stability was further investigated by monitoring the activity after treatment at different temperatures for 1 h and results are depicted in Figure 4.6. It was noticed that the XR was fully active and stable up to 40 ºC at pH 7.0. However, the enzyme lost 34.45% of its original activity at 50 ºC (retained 65.55% relative activity) and retained only 18.26% of its activity at 60 ºC. The activity of XR was fully lost at 80 ºC probably be due to the thermal denaturation of the enzyme or coenzyme NADPH (Figure 4.6). It has been reported that NADPH gradually became inactive when incubated at temperatures higher than 40 ºC (Wu et al., 1986).

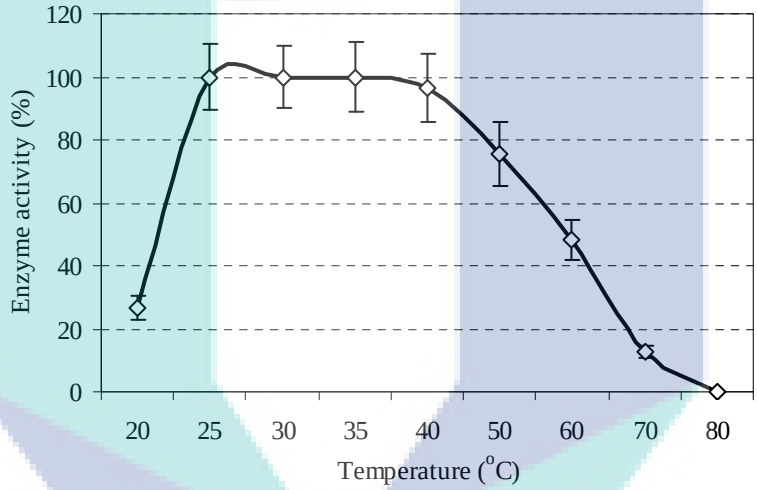


Figure 4.5: Optimum temperature for XR activity

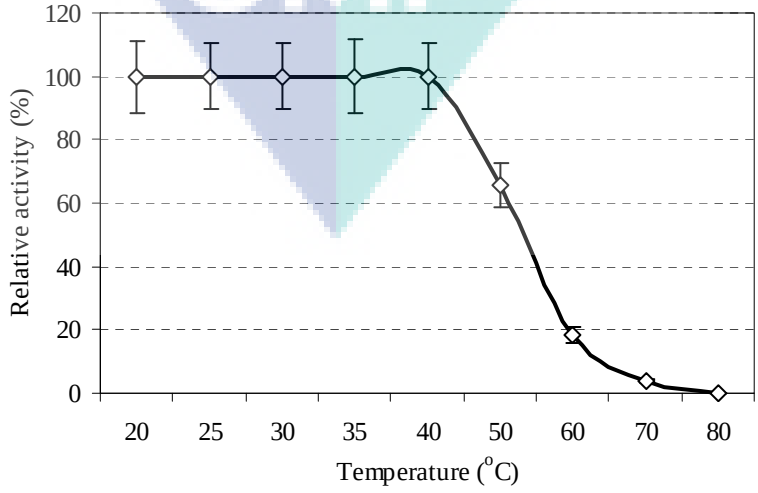


Figure 4.6: Thermal stability of XR

Effect of Incubation Period on XR Activity and Stability

The influence of incubation period on XR stability was evaluated by measuring the residual activity at various intervals (at 25 ºC and pH 7.0) and expressed as relative activity after incubation at different period of time (1–48 h). The results are presented in Figure 4.7. It was observed that XR did not lose any activity up to 24 h of incubation. The activity of XR gradually decreased with further increase in incubation period (>24 h) and reduced drastically at 48 h of incubation, retaining only 19.33% relative activity (Figure 4.7). This decline in enzyme activity and stability during incubation might be attributed to protein denaturation or to degradation by proteases present in the crude XR extract. Similar results have been reported for XR obtained from different yeasts of *Candida* genus (Cortez et al., 2006; Ronzon et al., 2012 and Sene et al., 2001).

Effect of Storage Time on XR Activity and Stability

The analysis of storagability of XR was carried out up to 120 days (4 months) and its remaining activity was measured at 30 days intervals. The results of XR storage stability are furnished in Table 4.2. The XR remained stable at –80 ºC for 90 days and there was a slight decrease in activity (4.75% of the original activity) after 120 days of storage (Table 4.2). This might be due to the chilling effect, a usual phenomenon for protein (Boscolo et al., 2009 and Cui et al., 2008).

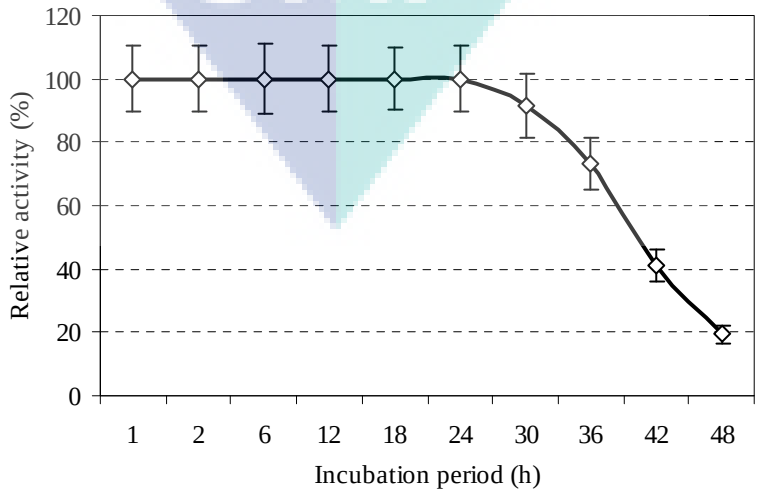


Figure 4.7: Effect of incubation period on XR activity and stability

Table 4.2: Effect of storage time on XR activity and stability

Storage time	Original XR activity (U/mL)	Remaining activity (U/mL)	Relative activity[a] (%)
0 day	11.16	11.16	100
30 days		11.16	100
60 days		11.16	100
90 days		11.09	99.37
120 days		10.63	95.25

[a] Relative activity = (remaining activity)/(original activity) × 100

Kinetic Parameters of Xylitol Production

The kinetic parameters of XR, K_m and V_{max}, were determined to investigate their effect on xylitol production in batch system. The K_m and V_{max} values were calculated using the Lineweaver-Burk plot at defined reaction conditions for both xylose and NADPH (at 12.25 h, 35 ºC, pH 6.5, 100 rpm and 3% of XR) mentioned in subsection 3.7.10. During xylose reduction by XR, initial velocity was measured at varied D-xylose concentration, keeping NADPH concentration constant (300 µM), while it was measured at varied NADPH concentration, keeping D-xylose constant (280 mM). The values of kinetic parameters obtained from the Lineweaver-Burk plot are presented in Figure 4.8. The obtained velocities yielded straight converging lines that intersected to the left of the ordinate for the double-reciprocal plot of initial velocity against varied substrate concentration (Figure 4.8). These findings implied that the Michaelis-Menten constant (K_m) for one substrate is dependent on the concentration of the other substrate. The K_m values of XR for xylose and NADPH were 81.78 mM and 7.29 µM with the corresponding V_{max} for xylose and NADPH of 178.57 and 12.5 µM/min, respectively. The K_m for xylose and NADPH obtained in this work are consistent with other researchers (Neuhauser et al., 1997 and Verduyn et al., 1985b) who reported those ranging from 14–72 mM and 4.8–21 µM, respectively. It is noticeable that higher V_{max} and lower K_m of XR for xylose are associated with the greater xylitol production. In addition, the lower K_m value of xylose reflects that XR has a higher affinity to its substrate xylose. In other words, xylose exhibited strong binding with XR. The higher value of V_{max} proved that this reaction would produce a higher concentration of xylitol.

The experimental data of the kinetic studies on xylose reduction by XR are listed in Appendix B (Table B.2).

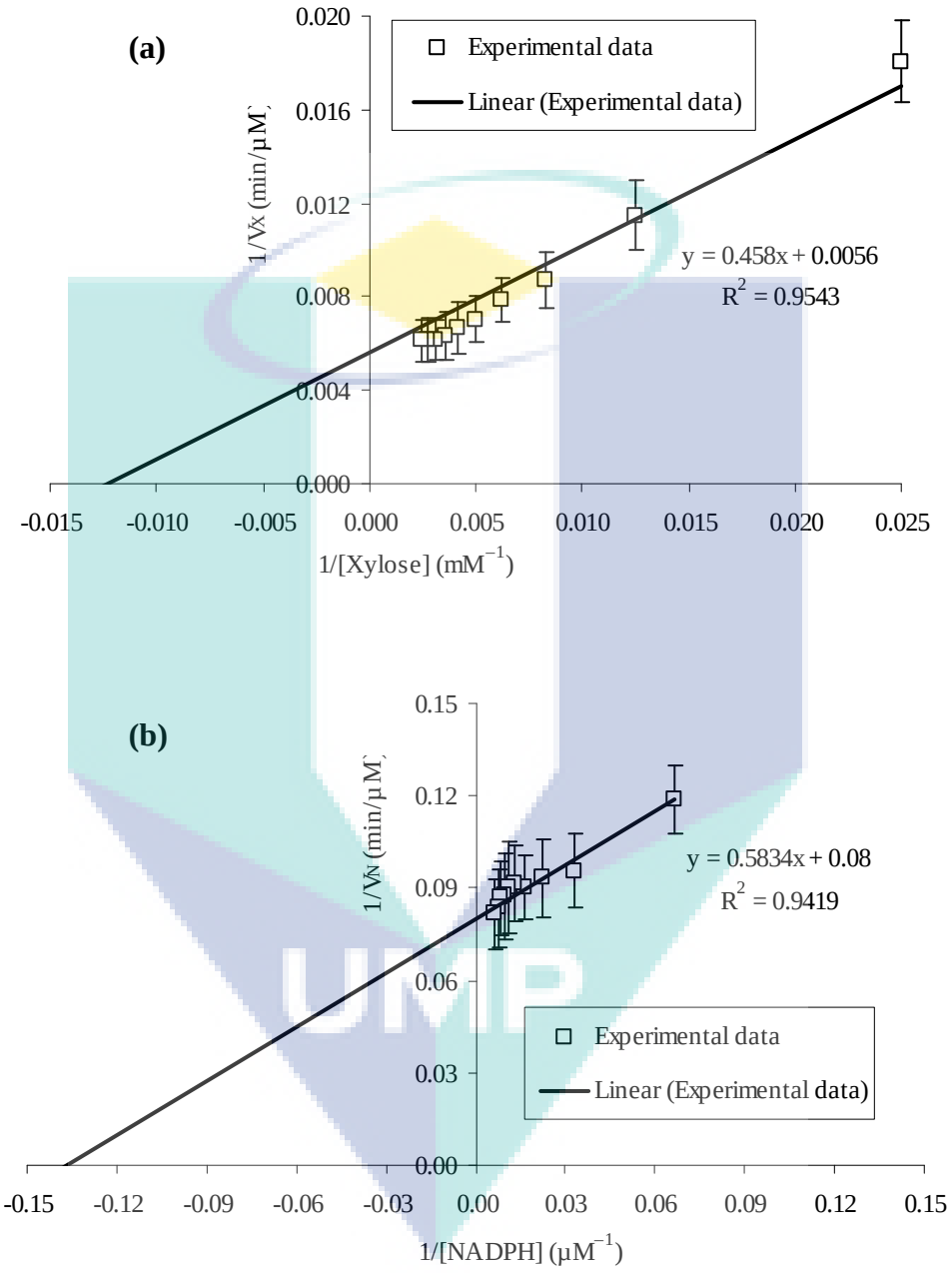


Figure 4.8: Lineweaver-Burk plot for the determination of K_m and V_{max} for (a) xylose and (b) NADPH. V_X and V_N are the initial reaction velocities of XR for xylose and NADPH, respectively

MIC for XR Inhibition

XR, a biocatalyst that catalyzes the conversion of xylose to xylitol can also be affected by inhibitors and consequently the rate of xylitol production might decrease. It is, therefore, important to assess the inhibitory impacts of various undesired compounds present in the hemicellulosic hydrolysates on XR in order to improve xylitol production. In this study, minimum inhibitory concentration (MIC) assays were carried out to identify the selected byproducts in the MWSHH inhibiting XR activity *in vitro* and to quantify their inhibitory effects. The compounds tested and compared for their inhibitory effects were glucose, arabinose, acetic acid, furfural, HMF, and tannic acid (that is a small lignin degradation model compound). These compounds have been frequently found in varying amounts in the hydrolysates of different LCMs (Mussatto and Roberto, 2005 and Villarreal et al., 2006). A large number of studies have been performed on the inhibitory effects of these byproducts on microbial xylitol production and on their inhibitory mechanisms (Arvela et al., 2011; Mussatto and Roberto, 2005; Parajó et al., 1995; Villarreal et al., 2006 and Zhang et al., 2012). It was reported that in high contents, acetic acid, furfural, HMF, and LDPs are able to individually and synergistically hinder microbial metabolism, impair xylose fermentation and thus lower xylitol yield (Parajó et al., 1998b and Zhang et al., 2012). But there are no reports, so far, on the *in vitro* inhibitory effect of these byproducts on XR activity.

The activity of an inhibitor or antimicrobial agent against enzymes or microorganisms is typically measured by MIC assay. MIC is the most widely accepted and basic laboratory bioassay method because of its technical simplicity and capability to quantify a particular compound's inhibitory effect (Sabitha et al., 2012; Turnidge et al., 2003 and Wadhwani et al., 2009). More recently, Nickavar and Yousefian (2011) reported that MIC is successfully used to evaluate whether a specific compound has any influence on the inhibition associated with an enzyme activity. In addition, this assay is useful as a screening approach while there are many compounds to test simultaneously (Turnidge et al., 2003). The inhibitory effect of each byproduct in the MWSHH is presented in Table 4.3.

Table 4.3: Inhibitory effects of the minor compounds present in the MWSHH on XR (in terms of MIC and IC_{50})

Test compounds	Concentration of compounds (g/L)	XR added (mL)	Inhibition (%)	MIC (g/L)	IC_{50} (g/L)
Glucose	10	0.1	4.6 ± 0.61	10	ND
	5	0.1	NI		
	2.5	0.1	NI		
	1.25	0.1	NI		
	0.625	0.1	NI		
Arabinose	5	0.1	NI		ND
	2.5	0.1	NI		
	1.25	0.1	NI		
	0.625	0.1	NI		
	0.313	0.1	NI		
Acetic acid	8	0.1	24.6		11 ± 1.31
	4	0.1	7 ± 0.88	4	
	2	0.1	NI		
	1	0.1	NI		
	0.5	0.1	NI		
Furfural	1.5	0.1	37		2.3 ± 0.28
	0.75	0.1	10 ± 1.48	0.75	
	0.375	0.1	NI		
	0.188	0.1	NI		
	0.094	0.1	NI		
HMF	0.2	0.1	28.2		0.4 ± 0.05
	0.1	0.1	8 ± 0.91	0.1	
	0.05	0.1	NI		
	0.025	0.1	NI		
	0.013	0.1	NI		
Tannic acid	4	0.1	17.5		6.4 ± 0.69
	2	0.1	5 ± 0.46	2	
	1	0.1	NI		
	0.5	0.1	NI		
	0.25	0.1	NI		

NI = no inhibition, ND = not determined

The presence of glucose in the reaction medium at concentrations of 0.625–5 g/L did not affect XR activity, but glucose at 10 g/L was found to inhibit only 4.6% of its original activity. Thus, the MIC of glucose for XR determined was 10 g/L. Arabinose did not exhibit any inhibitory effect on XR under the ranges investigated (0.312–5 g/L). The MIC values of acetic acid, furfural, HMF, and tannic acid against XR activity measured were 4 (inhibited by 7%), 0.75 (inhibited by 10%), 0.1 (inhibited by 8%), and 2 (inhibited by 5%) g/L, respectively. It is highlighted that the byproducts content in the MWSHH showed insignificant inhibitory effect on XR activity (inhibition <15%). Nidetzky et al. (1996) reported that the inhibitory effect of substrate, products or other components was considered significant when more than 15% of inhibition is observed. Thus, the crude MWSHH can be used as xylose source for the enzyme-based *in vitro* production of xylitol.

It was evident that XR inhibitory activities varied among the tested compounds. Based on the MIC values, acetic acid, furfural, HMF, and LDPs are considered as the potent inhibitor in the MWSHH for XR activity. Hence, the concentration dependent XR inhibitory activities of these compounds were further examined and their IC_{50} values were calculated. The inhibitory concentration–50 **(**IC_{50}) value is defined as the concentration of a test compound required to inhibit 50% of XR activity under standard assay conditions (Nagai et al., 2012 and Sabitha et al., 2012). A lower IC_{50} value reflects greater inhibitory effect of the compound on enzyme activity (Haripyaree et al., 2010). According to the dosage, all of these compounds exhibited a significant reduction in the XR activity. HMF showed the highest inhibitory effect with an IC_{50} value of 0.4 g/L (Table 4.3) and it might be due to the direct hindrance of enzyme action or coenzyme consumption by HMF. The IC_{50} values of acetic acid, furfural, and LDPs were 11, 2.3, and 6.4 g/L, respectively. The IC_{50} of glucose and arabinose were not determined because of their insignificant inhibitory effect on XR. It was observed that there is insignificant synergistic inhibitory effect (inhibited by 13.8%) on XR activity when all the inhibitors are present at a time in the reaction mixture (at concentrations of 4.64 g/L glucose, 2.55 g/L arabinose, 4.14 g/L acetic acid, 0.55 g/L furfural, 0.08 g/L HMF, and 1.55 g/L tannic acid).

The results of MIC studies indicated that HMF is the more potent inhibitor for XR (based on IC_{50} values) followed by furfural, LDPs, and acetic acid (i.e., HMF > furfural > LDPs > acetic acid), which are consistent with the reported information regarding the inhibition of fermentation organism's growth and xylitol production by these compounds (Guo et al., 2008; Mussatto and Roberto, 2005 and Parajó et al., 1998b). Hence, the MIC assay is viable in screening the toxic compounds that can reduce enzyme activity and consequently can decrease xylitol yield. Furthermore, the analysis of inhibitory effect of individual byproducts using MIC is important in order to formulate hydrolysate production from LCMs. These results validated the use of raw hemicellulosic hydrolysate for xylitol synthesis by XR and reported for the first time on the inhibitory activities of several byproducts for XR.

The stability studies showed that XR was stable in the pH range from 6.0–7.0 at temperature of 25ºC for 24 h, and retained above 95% of its original activity after 4 months of storage at –80 ºC. The activity and stability of XR obtained in this study seem to be promising for the efficient production of xylitol using MWSHH as a source of xylose in the enzymatic conversion. XR exhibited Michaelis-Menten kinetics with respect to its substrates namely xylose and NADPH. The enzymatic reaction between XR and xylose is highly productive because of the high V_{max} and low K_m values. Hence, detail knowledge of the xylitol production kinetics in multi-substrate enzymatic reaction could help in the design of processes for large-scale production of xylitol from lignocellolusic biomass. MIC study demonstrated that the high levels of decomposition compounds (acetic acid, furfural, HMF, and LDPs) have independent inhibitory impacts on XR activity and xylitol synthesis, but they may also have synergistic inhibitory impacts. Thus, it warrants further study to analyze the synergistic inhibitory effects of multiple byproducts on XR activity and to eliminate the toxic compounds from the concentrated hemicellulosic hydrolysates for the improvement in xylitol yield.

4.3 Conclusion

MWS is a cheap and widely available lignocellulosic biomass. The characterization of MWS biomass would play a vital role in a lignocellulose-based biorefinery industry for processing of its main constituents (cellulose, hemicellulose and

lignin) to various bioproducts and biofuels. It contains 29.22% xylan, which is a promising source of xylose. This xylose can be used as a potential carbon source to culture microbes, and to produce a wide variety of specialty chemicals, mainly xylitol. Conversion of hemicellulosic fraction of MWS waste to sugars provides a feedstock for the manufacture of high value products and will substantially reduce the amount of waste that would otherwise exert pressure on landfills as well as environment pollution.

The economic interest in utilizing xylose as carbon source for the production of XR from xylose-fermenting organisms would be enhanced if the growth media could be made from hemicellulosic hydrolysate of LCMs instead of using commercial xylose. The XR isolated from *C. tropicalis* grown in MWS hydrolysate medium was specific to NADPH as coenzyme with the activity of 11.16 U/mL. XR showed the K_m values of 81.78 mM and 7.29 μM, and V_{max} of 178.57 and 12.5 μM/min for xylose and NADPH, respectively. The high V_{max} (178.57 μM/min) and low K_m (81.78 mM) values of XR for xylose implying a highly productive reaction among XR and xylose. The byproducts detected in the MWSHH (such as glucose, arabinose, acetic acid, furfural, HMF, and LDPs) were subjected to MIC assay to determine their degree of inhibition on XR activity. The IC_{50} values of acetic acid, furfural, HMF, and tannic acid for XR were 11, 2.3, 0.4, and 6.4 g/L, respectively. The presence of glucose and arabinose were not inhibitory to XR up to a concentration of 5 g/L. In this study, MWSHH proved to be an important alternative carbon and energy source for cultivating the yeast *C. tropicalis*. This yeast also proved to be a potential source of intracellular enzyme XR when cultured on hydrolysate-based medium. The XR prepared in the laboratory can be used as biocatalyst for the *in vitro* production of various commercially important products including xylitol. The utilization of MWSHH as a source of xylose will not only reduce the application of commercial xylose but also prepare an industrially important enzyme XR from hemicellulosic hydrolysate of sawmill's waste.

CHAPTER 5

RECOVERY OF XYLOSE FROM MWS

This chapter summarizes the results and discussion on the recovery of xylose. To provide a clear understanding of the research work, the results and the discussion are arranged in accordance with the following sequences: The beginning of this chapter focused on the design of process factors for xylose recovery from MWS by acid hydrolysis. A rough screening of suitable ranges of process factors for MWS hydrolysis by OFAT and their subsequent refinement through kinetic studies are highlighted. The optimization of xylose recovery from MWS using RSM is presented at the end of this chapter. This chapter also provides a detailed discussion on each step and the observed results are validated through literature survey.

5.1 INTRODUCTION

Biotechnological production of biofuels, specialty chemicals or food ingredients from lignocellulosic biomass has attracted considerable attention because the biomass is renewable, widespread and inexpensive as a source of polysaccharides (Kuhad and Singh, 1993; Parajó et al., 1996 and Rahman et al., 2007). Sawdust is a cheap lignocellulosic waste of sawmill that is available throughout the year. The hydrolysis of *Meranti* wood sawdust (MWS) to produce xylose solution could be a good alternative to manage this abundant waste. Moreover, the utilization of MWS to produce xylose has a dual benefit, the elimination of waste and the generation of high value product. Xylose can be an economical starting raw material for the production of a wide variety of specialty chemicals or fuels by chemical and biotechnological processes. One of these

specialty chemicals is xylitol that is extensively utilized in the food, pharmaceutical, cosmetic, and odontological industries (Roberto et al., 1995, 2003).

Numerous investigations on dilute acid hydrolysis of different lignocellulosic materials (LCMs) such as *Eucalyptus* wood, corncobs, sugarcane bagasse, sweet sorghum bagasse, brewer's spent grain, oil palm empty fruit bunch, and sorghum straw have been performed by several research groups (Canettieri et al., 2007; Dominguez et al., 1997; Lavarack et al., 2002; Liu et al., 2012; Mussatto and Roberto, 2005; Rahman et al., 2007 and Téllez-Luis et al., 2002). It was reported that xylose was produced as the main sugar from hemicellulose and at the same time, other byproducts such as glucose, arabinose, acetic acid, furfural, hydroxymethylfurfural (HMF), and lignin degradation products (LDPs) were also generated in low amounts during hydrolysis (Dominguez et al., 1997; Liu et al., 2012 and Mussatto and Roberto, 2005). It was also reported that the amount of sugar released during hydrolysis depended on the type of raw material and operating conditions of the experiment such as temperature, acid concentration and residence time (Pessoa Jr et al., 1996). During hydrolysis, acid concentration was found to be the most important parameter affecting the sugar yield while temperature showed the highest impact on the formation of sugar degradation products (Neureiter et al., 2002). Under controlled experimental conditions, dilute acid hydrolysis of lignocellulosics mainly produces xylose from hemicellulose, leaving a solid residue containing the cellulose and lignin fractions almost unaltered. Hemicelluloses are more susceptible to mild acid due to its amorphous, branched structure compared to cellulose, which needs severe treatment conditions due to its crystalline nature (Chundawat et al., 2011 and Parajó et al., 1998b).

The MWS biomass contains cellulose, hemicellulose and lignin as the major biopolymers. It is estimated that this biomass is comprised of more than 29% xylan, a sugar polymer made of pentose sugar xylose (Table 4.1 of chapter 4). The hemicellulosic fraction can be easily and selectively extracted with dilute sulfuric acid under mild conditions to obtain xylose-rich hemicellulosic hydrolysate which can be used as a substrate to produce xylitol by bioconversion. The acid hydrolysis of MWS can also lead to a valuable solid residue mainly formed from cellulose and lignin. This residue can be utilized in the production of glucose which, in turn, can be used for the

generation of ethanol, lactic acid, or for making high grade paper. Hence, the acid hydrolysis can be conceived as the first step of an integrated strategy for sawdust utilization. Dilute acid hydrolysis is still preferable to enzymatic hydrolysis as it is low cost, simple, faster method and commonly used for the hydrolysis of lignocellulosic biomass (Carvalheiro et al., 2005; Liu et al., 2012 and Rivas et al., 2006). However, the major disadvantage of acid hydrolysis is that it generates a hydrolysate that contains not only the sugar needed for bioconversion but also sugar and lignin degradation products as well as acetic acid that could slow down or prevent the bioconversion of xylose (Parajó et al., 1995). Therefore, it is important to run the hydrolysis reaction at less severe conditions and optimize the operational conditions in order to ensure high xylose recovery and low byproducts generation.

There is no report available on the acid hydrolysis of wood sawdust to extract xylose. The objective of the present experiment was to optimize the hydrolysis process and determine the effect of temperature, sulfuric acid concentration, and residence time on the recovery of xylose and the formation of byproducts (glucose, arabinose, acetic acid, furfural, HMF, and LDPs) from MWS. To obtain the optimum conditions, this study was conducted in three stages: firstly, the one-factor-at-a-time (OFAT) approach was applied to design the process factors affecting xylose recovery for further optimization. Secondly, the parameters of kinetic models were determined to predict the production of xylose, glucose, furfural, and acetic acid during hydrolysis of MWS. Thirdly, the central composite design (CCD) under response surface methodology (RSM) was used to achieve the actual optimum conditions by developing a quadratic model in order to attain high xylose yield and selectivity.

5.2 RESULTS AND DISCUSSION

5.2.1 Design of Parameters for Xylose Recovery

Parameter design is a method of selecting nominal values for the set of operating variables. It can be undertaken to optimize robustness or to optimize the nominal response (Frey et al., 2003). Conventionally, parameter design and/or optimization of a chemical process is carried out by monitoring the influence of one variable at a time for

an experimental response. This optimization technique is called one-factor-at-a-time (OFAT) that involves changing the levels of one factor while keeping all others constant (Bezerra et al., 2008 and Tinoi et al., 2005). In an OFAT approach, a researcher seeks to get information about one parameter in each experimental trial and this procedure is repeated by turn for all factors to be investigated (Frey et al., 2003). OFAT is a simple and straight forward experimental design, which does not require advanced statistical knowledge. However, this classical method is time-consuming and incapable of detecting the true optimum conditions, especially due to the absence of the interaction effects among the factors studied (Liu and Tzeng, 1998 and Montgomery, 2001). MWS hydrolysis conditions were primarily optimized by the traditional OFAT approach for maximum xylose recovery and minimum byproduct formation. The objective of the parameter design experiment was to study the effect of residence time, temperature, sulfuric acid concentration, and liquid to solid ratio (LSR) on the formation of xylose and byproducts (glucose, arabinose, acetic acid, furfural, HMF, and LDPs) from MWS and to design the process parameters for further optimization. The experimental strategy that is used extensively is OFAT method to design the process variables (Montgomery, 2001). In the first step of optimization process, this method was followed to observe the effective range of factors for xylose extraction as no specific information was available in the literature regarding xylose production from wood sawdust. The influences of 4 operational parameters (residence time, temperature, H_2SO_4 concentration, and LSR) on the hydrolysis of MWS hemicellulose, evaluated by the OFAT method, are detailed in the following subsections.

Effect of Residence Time

Different levels of residence time ranging from 10–120 min were tested to determine the possible optimum value for maximum xylose and minimum byproducts formation. Figure 5.1 shows the effect of residence time on the formation of xylose and other byproducts at constant LSR (8 g/g), H_2SO_4 concentration (2%), and temperature (130 ºC). From the figure it was evident that the concentration of xylose and arabinose in the resulting hydrolysate increased with increase in residence time to certain extent and then decreased with further increase of residence time. The highest xylose and arabinose concentration of 14.75 and 2.47 g/L, respectively, were obtained by

conducting hydrolysis for 60 min keeping other parameters constant (Table 3.1). The reduction in xylose and arabinose concentration at longer residence time (>60 min) resulted from further degradation of these compounds to furfural. Torget et al. (1991) reported that xylose and arabinose decomposition is proportional to reaction time and acid concentration. A considerable amount of xylose was released within 40–80 min of residence time by acid hydrolysis of MWS. Thus, the possible optimum residence time was 60 min that was subsequently optimized by RSM. The concentration of glucose also increased with increasing residence time, the highest concentration of 6.22 g/L being obtained at 120 min. Figure 5.1 clearly shows that the concentration of acetic acid, furfural, HMF, and LDPs steadily increased with increase in residence time, achieving a maximum value of 4.52, 0.73, 0.14, and 3.1 g/L, respectively, for 120 min. Shorter reaction time reduces the release of glucose and generation of furfural (Herrera et al., 2003). This 60 min of residence time was selected for further experiments to observe the effect of temperature, acid concentration and LSR. The OFAT experimental plan and composition of MWS hydrolysate obtained in each run are summarized in Appendix B (Table B.3).

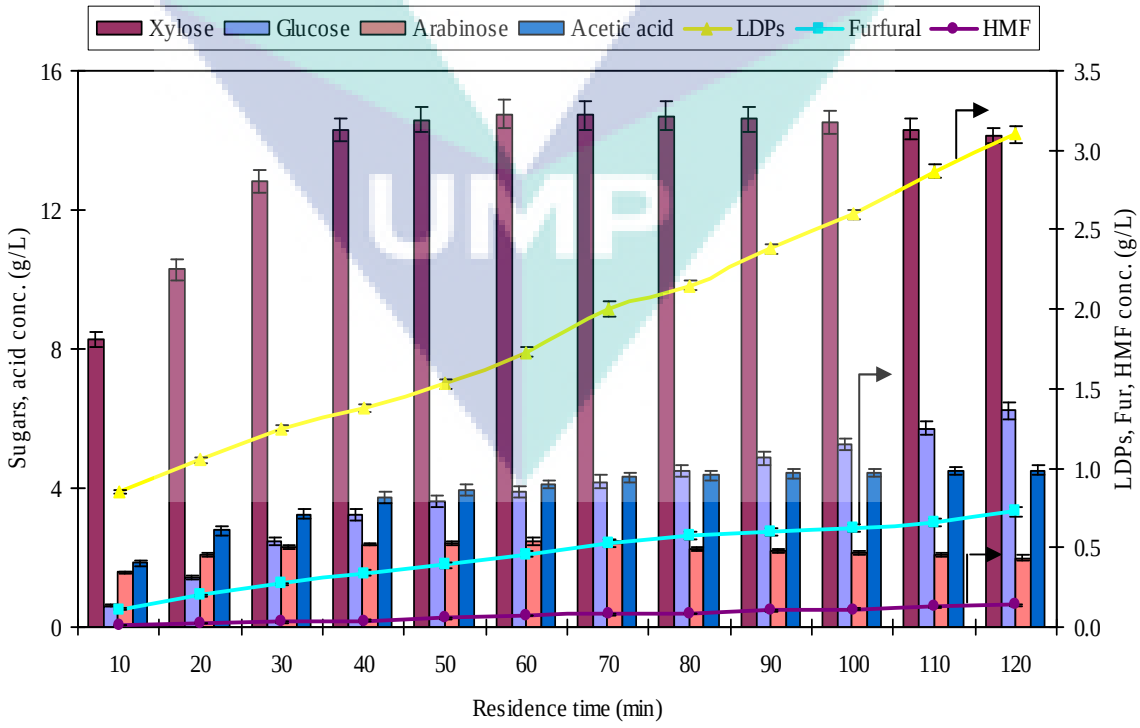


Figure 5.1: Effect of residence time on the formation of xylose and other byproducts at constant LSR 8 g/g, H_2SO_4 concentration 2%, and temperature 130 ºC

Effect of Temperature

To evaluate the effect of temperature on the production of xylose, six temperature levels varying from 105–130 ºC were employed, keeping other parameters constant. The data are presented in Figure 5.2. Xylose concentration in the hydrolysate increased with increasing temperature and reached to a maximum value of 14.78 g/L at 125 ºC when other factors were fixed (Table 3.1). Similar trends were observed for all other byproducts. The concentration of xylose slightly decreased on further increase of temperature (14.75 g/L at 130 ºC) while the undesired products (glucose, arabinose, acetic acid, furfural, HMF, and LDPs) significantly increased (Figure 5.2). The result revealed that the hemicellulosic fraction depolymerizes faster at lower temperature than the cellulose fraction with dilute acid treatment while at higher temperature or longer retention time, the formed monosaccharides further hydrolyzes to other compounds (Karimi et al., 2006). The highest level of temperature was fixed at 130 ºC because xylose concentration was found to decline slightly at this temperature and it was the maximum temperature attainable by the autoclave used. Temperature between 120 and 130 ºC was critical to xylose recovery with the observed optimum value of 125 ºC which was applied in the subsequent experiments.

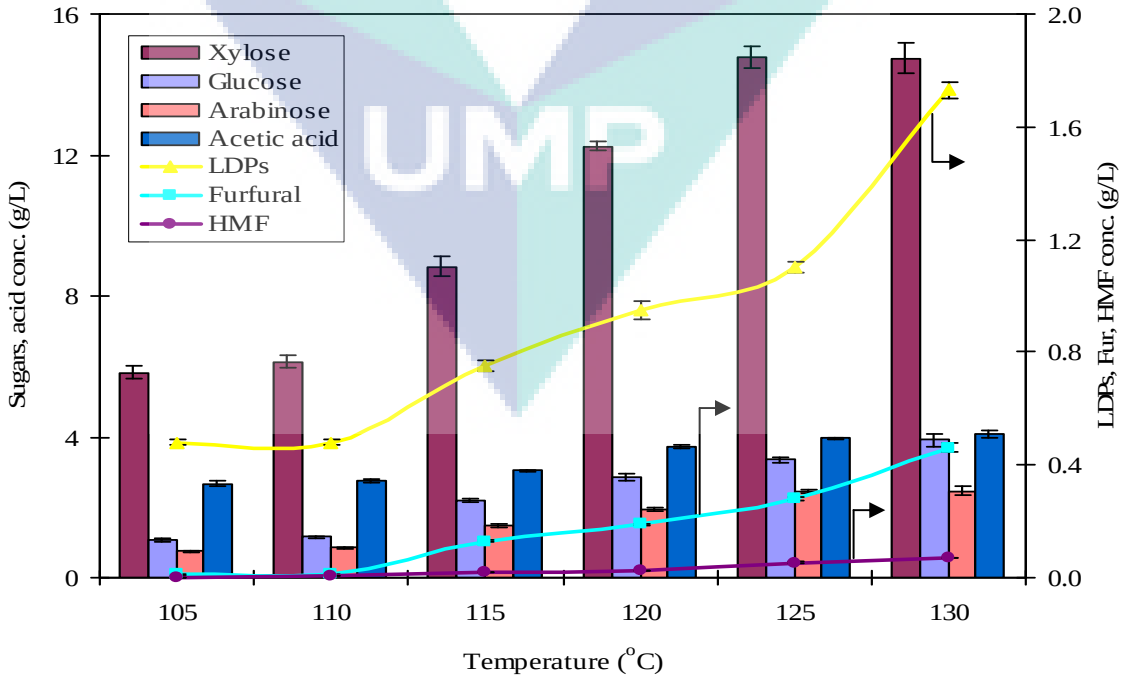


Figure 5.2: Effect of temperature on the formation of xylose and other byproducts at constant LSR 8 g/g, H_2SO_4 concentration 2%, and residence time 60 min

Effect of H_2SO_4 Concentration

The influence of sulfuric acid concentration was determined by varying its concentration from 2–12% (w/w). The concentration of xylose rapidly increased from 14.78 to 17.90 g/L with an increase of acid concentration from 2 to 4% while other parameters were kept constant (Figure 5.3 and Table 3.1), whereas arabinose concentration increased slightly (2.44 to 2.59 g/L). It was observed that xylose and arabinose concentration did not increase further until 6% acid and a further increase in acid concentration led to a sharp decline of these compounds (Figure 5.3). This reduction in xylose concentration may be attributed to the degradation of xylose to furfural. These results are consistent with the previous investigation that reported xylose to be more sensitive to degradation to furfural compared to glucose, particularly at acid concentrations above 1% and temperature above 120 ºC (Baek and Kwon, 2007). Figure 5.3 demonstrates that the amounts of glucose, acetic acid, furfural, HMF, and LDPs in the resulting hydrolysate increased as the acid concentration increased. The observed optimum acid concentration was 4% that was further optimized by RSM.

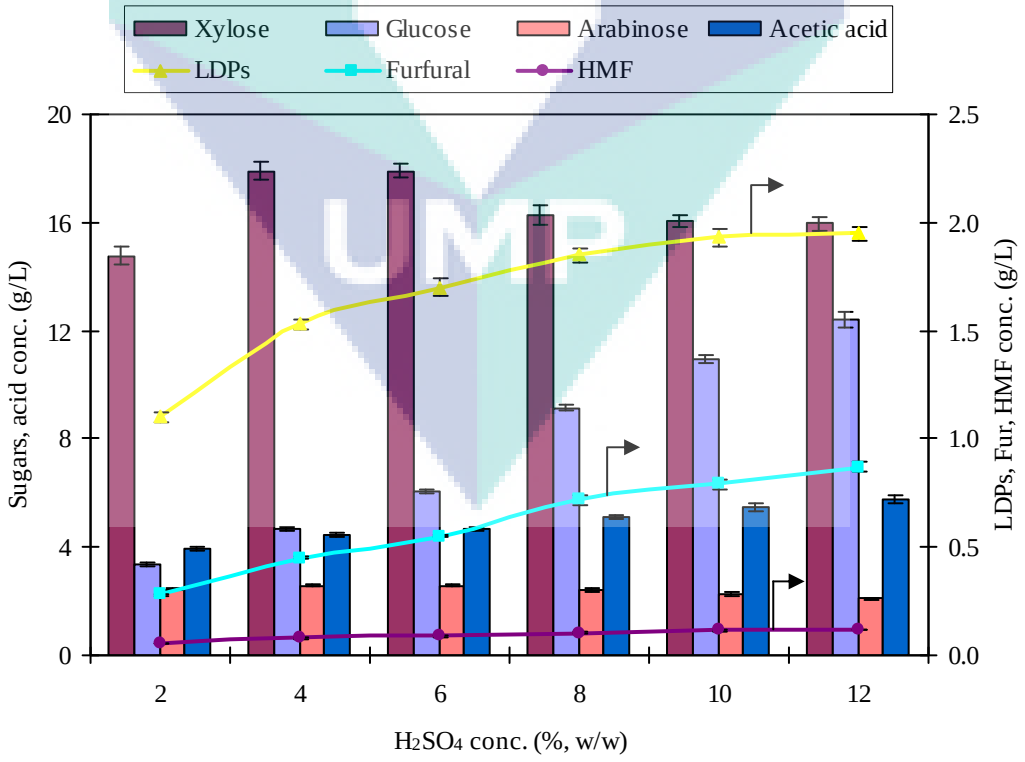


Figure 5.3: Effect of H_2SO_4 concentration on the formation of xylose and other byproducts at constant LSR 8 g/g, time 60 min, and temperature 125 ºC

Effect of Liquid to Solid Ratio (LSR)

Various levels of LSR were investigated to determine the optimum LSR for the maximum recovery of xylose. Figure 5.4 shows the effect of various LSR on the formation of xylose and other byproducts at constant values of H_2SO_4 concentration (4%), residence time (60 min), and temperature (125 ºC). The highest xylose concentration achieved was 17.9 g/L with a LSR of 8 g/g. This result was similar to a previous report (Mussatto and Roberto, 2005). The xylose concentration dropped significantly with further increase in LSR, indicating that the reduced xylose concentration is likely to result from the lowest solid content. Similar trends were observed for the concentrations of all byproducts generated during acid hydrolysis. Dominguez et al. (1997) showed that the highest amount of xylose was obtained with the highest solid content. The amount of acid used per unit weight of solid can be reduced by performing hydrolysis at lower LSR. Moreover, the lower LSR is more acceptable due to lesser water requirement.

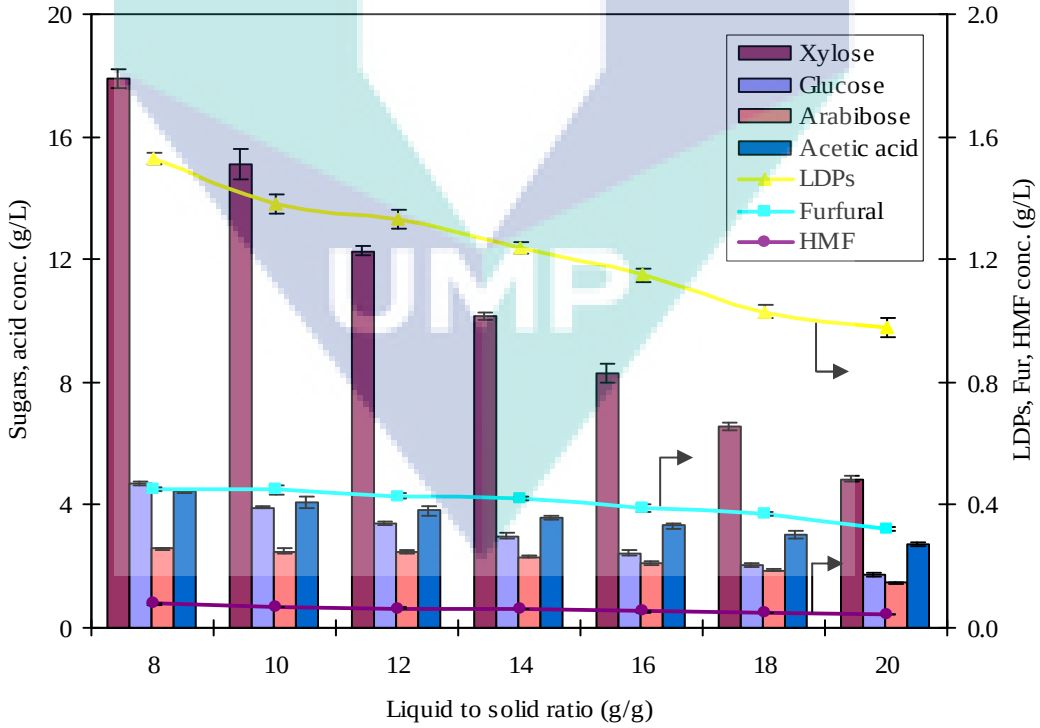


Figure 5.4: Effect of LSR on the formation of xylose and other byproducts at constant H_2SO_4 concentration 4%, residence time 60 min, and temperature 125 ºC

The observed optimum residence time, temperature, acid concentration, and LSR found by OFAT method were 60 min, 125 ºC, 4%, and 8 g/g, respectively. These conditions yielded a hemicellulosic hydrolysate containing 17.9 g/L xylose (which is 86.3% of the PC of xylose present in the MWS), 4.69 g/L glucose, 2.59 g/L arabinose, 4.45 g/L acetic acid, 0.45 g/L furfural, 0.08 g/L HMF, and 1.53 g/L LDPs. The LSR had no significant effect on xylose recovery; thus the treatment at its lower level (8 g/g) was preferred and selected for the subsequent studies because of its low acid and water consumption. The OFAT study demonstrated that the recovery of xylose from MWS hemicellulose were influenced by temperature, acid concentration, and residence time alone, which are consistent with the previous research report (Pessoa Jr et al., 1996). Thus, the effect of these independent variables was further analyzed by CCD to optimize the hydrolysis process for achieving maximum xylose output with minimum undesired products. Therefore, parameter design through OFAT proves to be a key step of optimization studies to obtain high yield and/or quality of product without increasing the cost.

5.2.2 Kinetic Studies on Acid Hydrolysis of MWS

The simplest kinetic model of hemicellulose hydrolysis represents that xylan is initially converted to xylose, which in turn is decomposed to furfural. Since differences in structure and composition of biomass affect reaction rate, it is important to determine the kinetic parameters of each raw material. The aim of the kinetic study was to determine the parameters of kinetic models for predicting the production of xylose, glucose, furfural, and acetic acid during hydrolysis of MWS by sulfuric acid and to optimize the process. In order to evaluate experimental conditions for optimizing xylose yields, it is necessary to know the kinetic characteristics of products released during acid hydrolysis of MWS. The findings of kinetic studies on acid hydrolysis of MWS for the recovery of xylose are addressed in the following subsections.

Composition of MWS Hydrolysate

The maximum recovery of xylose from hemicellulose was 18.65 g/L, which was 89.9% of the potential concentration (PC) of xylose obtained with 4% sulfuric acid for 40 min of residence time and then decreased with increasing time (Figure 5.5A). The temperature of 130 ºC was chosen for all kinetic experiments on MWS hydrolysis because it is close to the optimal one (125 ºC) determined by OFAT study for the production of xylose (subsection 5.2.1) and to keep consistency with other reports (Parajó et al., 1994). On the other hand, the highest concentrations of xylose released were 18.53 and 14.75 g/L with 6% H_2SO_4 for 20 min and 2% H_2SO_4 for 60 min, respectively. The results demonstrated that in xylose release, the effects of H_2SO_4 concentration and residence time are inversely proportional. But higher acid concentration together with longer residence time adversely affect xylose yield leading to its breakdown to furfural. In another experiment using OFAT approach (subsection 5.2.1), it was found that the concentration of xylose rapidly increased from 14.78 to 16.91 g/L with an increase of acid concentration from 2% to 6% while other parameters were kept constant (temperature at 125 ºC, reaction time 60 min and LSR 8 g/g) and then decreased with further increase in acid concentration (16.28 g/L with 8% H_2SO_4). This reduction in xylose concentration may be attributed to its degradation to furfural. Under the same situation, the amount of undesired products in the hydrolysate increased with increasing acid concentration. Hence the observed highest acid concentration was selected to be 6%. The pH of the reaction mixture was not monitored throughout the experiment because the hydrolysis of MWS was carried out in an autoclave where pH monitoring was not possible. Only the initial and final pH was followed to be 0.28 and 0.71, respectively (with 6% acid for 20 min). The experimental and predicted data on the kinetic studies of MWS acid hydrolysis are given in Appendix B (Table B.4).

The dependence of experimental (as indicated by data points) and predicted (as indicated by solid lines) glucose release on the H_2SO_4 concentration and residence time at 130 ºC is shown in Figure 5.5B. During hydrolysis, glucose release was low at low acid concentration and reached to a maximum of 8.81 g/L with 6% H_2SO_4 for 120 min. Additionally, the highest concentrations of glucose generated were 7.7 and 6.22 g/L with 4% and 2% H_2SO_4, respectively, for 120 min. These results implied that glucose

concentration proportionately increased with acid concentration. The glucose in the hydrolysate can originate from both hemicellulose and cellulose polymer. Hence, the PC of glucose from glucan could not be determined. It is known that furfural is generated as a decomposition product from pentose sugars such as xylose and arabinose. Figure 5.5C shows the experimental and predicted values of furfural released during hydrolysis of MWS at 130 ºC. It was noted that when the acid concentration increased from 2% to 6%, furfural level also increased in the resulting hemicellulosic hydrolysate. The highest furfural concentration was found to be 1.82 g/L for 6% H_2SO_4 with a residence time of 120 min. It was also noted that furfural level continuously increased with acid concentration and reaction time. This result accorded with the reduction of xylose concentration at higher H_2SO_4 concentration, as observed in Figure 5.5A. During the treatment, acetic acid accumulates in the hydrolysate derived from acetyl groups of hemicellulose (Figure 5.5D). The highest and the lowest concentrations of acetic acid obtained were 5.03 and 2.78 g/L with 6% H_2SO_4 for 120 min and 2% H_2SO_4 for 20 min, respectively.

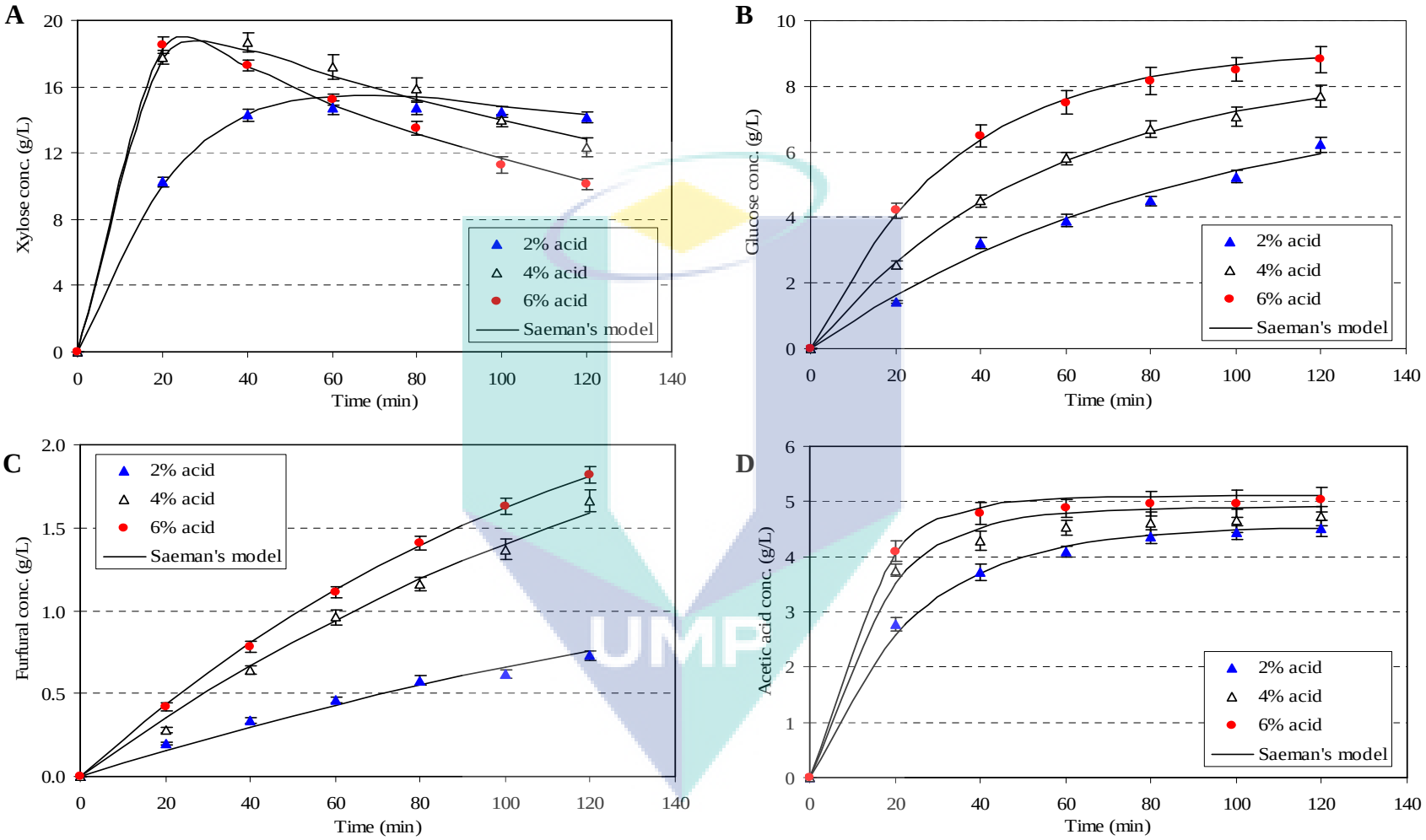


Figure 5.5: Dependence of experimental and predicted concentrations of components released during hydrolysis of MWS at 130 ºC on H_2SO_4 concentration and residence time: concentrations of (A) xylose, (B) glucose, (C) furfural, (D) acetic acid. Data points indicate experimental values and solid lines predicted values

Kinetic Model of Xylose Production

The initial xylan concentration [Xn_p], a value corresponding to the quantitative conversion from xylan to xylose, was calculated to be 20.75 g/L and the kinetic parameters were chosen by regression analysis. The experimental data were fitted to the Eq. (3.10) in order to obtain kinetic and statistical parameters. Experimental and predicted data for xylose recovery by MWS hydrolysis at 130 ºC with 2–6% sulfuric acid and 0–120 min residence time is shown in Figure 5.5A, and the kinetic and statistical parameters obtained for xylose at different acid concentrations is presented in Table 5.1. The data in Table 5.1 demonstrate that the rate of xylose formation (k_1) and breakdown (k_2) increased with increasing acid concentration. It was also noticed that the value of k_1 is about 26.5 fold higher than the k_2 values with 6% H_2SO_4. These findings suggested that the increasing H_2SO_4 concentration can shorten the residence time needed to obtain the highest recovery of xylose in the resulting hemicellulosic hydrolysate. The coefficient of determination R^2 showed a good agreement between experimental and model predicted results for all regressions.

Table 5.1: Kinetic and statistical parameters of components generated in the H_2SO_4 hydrolysis of MWS at 130 ºC

Products	2% H_2SO_4	4% H_2SO_4	6% H_2SO_4
Xylose			
k_1 (min^{-1})	0.03522	0.11788	0.16258
k_2 (min^{-1})	0.00381	0.00434	0.00614
R^2	0.9943	0.9961	0.9982
Glucose			
k_3 (min^{-1})	0.01134	0.0184	0.03006
[G_p] (g/L)	8.02	8.59	9.15
R^2	0.9890	0.9990	0.9992
Furfural			
k_4 (min^{-1})	0.0045	0.00621	0.00842
[F_p] (g/L)	1.81	3.03	2.86
R^2	0.9894	0.9957	0.9989
Acetic acid			
k_5 (min^{-1})	0.04188	0.06368	0.08012
[A_p] (g/L)	4.55	4.9	5.1
R^2	0.9973	0.9921	0.9985

Kinetic Model of Glucose Production

Glucose is the main byproduct in the MWS hydrolysate. The modeling of glucose formation was done using Eq. (3.12). However, the potential glucose concentration ([G_p]) could not be obtained experimentally as glucose proceeded from both hemicellulose and cellulose. Kinetic coefficient k_3 and [G_p] were obtained by regression. From Table 5.1 it was observed that the values of [G_p] were in the range of 8.02–9.15 g/L. These results agreed well with those reported for the sulfuric acid hydrolysis of sorghum straw. It was reported that the PC of glucose was in the range of 6.76–7.22 g/L (Téllez-Luis et al., 2002). The low concentration of glucose indicated that the cellulose fraction remained almost unchanged during acid hydrolysis and thus the glucose probably came from the hemicellulose. The values of k_3 were found to be in the range of 0.01134–0.03006 min^{-1}. It was also observed that the values of [G_p] and k_3 increased with increasing H_2SO_4 concentration, and the determination coefficient R^2 obtained was in good agreement with experimental and calculated data. Experimental and calculated data for glucose released in the hemicellulosic hydrolysate with various acid concentrations and residence time is shown in Figure 5.5B.

Kinetic Model of Furfural Production

Furfural is the principal degradation product of xylose during the acid hydrolysis of MWS. The actual and predicted data for furfural generated during hydrolysis of MWS at 130 ºC using several acid concentrations and residence time is shown in Figure 5.5C, and the kinetic and statistical parameters fitting the model for furfural are shown in Table 5.1. The PC of furfural [F_p] varied over the range of 1.81–3.03 g/L for each regression. These values are lower than the values found for the hydrolysis of sorghum straw (4 g/L) and oil palm empty fruit bunch fiber (OPEFB) (4.88–6.57 g/L) using sulfuric acid as a catalyst (Rahman et al., 2006 and Téllez-Luis et al., 2002). It was evident that the rate of furfural formation k_4 from both xylose and arabinose was only slightly higher than the rate of xylose decomposition to furfural k_2, implying that the conversion of arabinose to furfural was negligible. It was also evident that the kinetic constant k_4 increased with increasing the H_2SO_4 concentration. The R^2 values were well fitted with the furfural formation model (Table 5.1).

Kinetic Model of Acetic Acid Production

In the acid hydrolysis of MWS, acetic acid is liberated from acetyl groups bonded to the hemicellulosic sugars. Table 5.1 presents the kinetic and statistical parameters obtained for acetic acid generation at different concentrations of H_2SO_4. It was found that the values of [A_p] and k_5 were in the range of 4.55–5.1 g/L and 0.04188–0.08012 min^{-1}, respectively. The values of [A_p] are higher than the values obtained using sulfuric acid in the hydrolysis of sorghum straw (1.48–1.56 g/L), indicating that H_2SO_4 is a good catalyst to release acetyl groups present in the MWS biomass (Téllez-Luis et al., 2002). The R^2 showed that all the kinetic equations obtained for acetic acid were well fitted. It was also found that the kinetic constant k_5 increased with increasing concentration of H_2SO_4. Experimental and predicted data for acetic acid production during hydrolysis of MWS is shown in Figure 5.5D.

Generalized Kinetic Models

A generalized model was developed by correlating kinetic parameters with sulfuric acid concentration for predicting all product concentrations through the empirical Eq. (5.1), where i is an integer having values in the range of 1–5; k_0 and m are the regression parameters; C_a is the sulfuric acid concentration expressed in % (w/w).

$$k_i = k_0 C_a^m \tag{5.1}$$

In the generalized model, the rate of xylose release (k_1) and decomposition (k_2) were correlated with H_2SO_4 concentration (C_a) in the empirical Eqs. (5.2) and (5.3), respectively, as shown in Table 5.2. It was observed that the value of m for k_1 was 1.2453. The high values of R^2 were found for both parameters. Therefore, it is possible to predict xylose concentration at any residence time (0–120 min) and H_2SO_4 concentration (2–6%) by combining Eqs. (5.2) and (5.3) with Eq. (3.10).

Table 5.2: Generalized models for predicting kinetic parameters of MWS acid hydrolysis at 130 ºC

Products	Models	R^2
Xylose	$k_1 = 0.01778\, C_a^{1.2453}$ (5.2)	0.9918
	$k_2 = 0.00276\, C_a^{0.44}$ (5.3)	0.9897
Glucose	$k_3 = 0.00662\, C_a^{0.8135}$ (5.4)	0.9970
Furfural	$k_4 = 0.00288\, C_a^{0.59}$ (5.5)	0.9932
Acetic acid	$k_5 = 0.03003\, C_a^{0.5411}$ (5.6)	0.9880

The generalized model predicted that the highest xylose concentration obtainable were above 16, 17, and 19 g/L at 130 ºC with 2%, 4%, and 6% H_2SO_4 within 60, 40 and 20 min of residence time, respectively. The response surface graph of the generalized model for the prediction of xylose concentration with increase in H_2SO_4 concentration and residence time is shown in Figure 5.6. From the response surface it is possible to choose lower residence time in order to achieve the highest xylose concentration with minimum decomposition products in the hydrolysate. Moreover, by comparing response surfaces for several hydrolysis products, it is possible to achieve optimum conditions of the hydrolysis process.

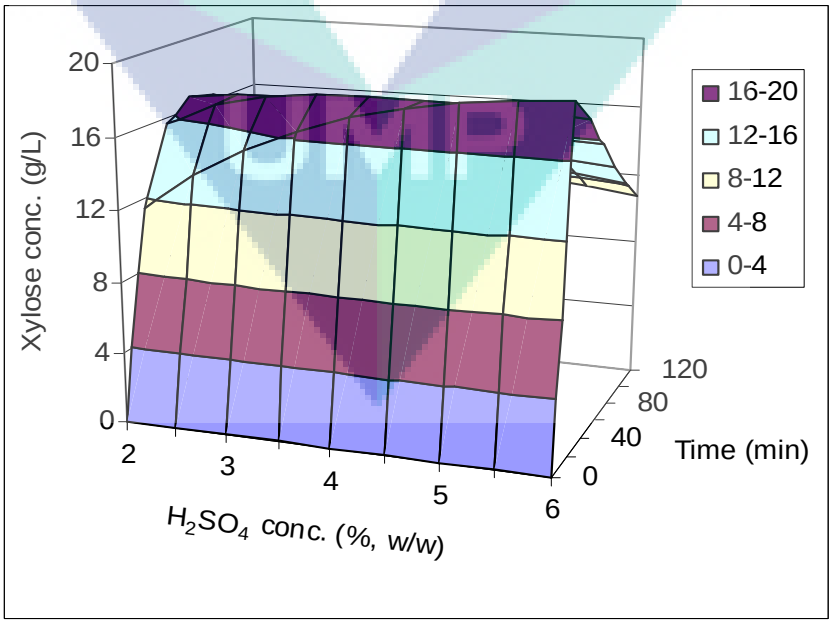


Figure 5.6: Influence of H_2SO_4 concentration and residence time on generalized model constructed for predicting xylose concentration

A generalized model was obtained to correlate kinetic parameter k_3 with C_a for predicting glucose concentration at any residence time and acid concentration within the range of operating conditions. The empirical Eq. (5.4) represents the generalized model of glucose production and the determination coefficient R^2 for k_3 fitted well as shown in Table 5.2. The value of m for k_3 was lower than the value of m for k_1 (Table 5.2), suggesting that heterogeneous reaction probably occurred during glucose generation from glucan. The influence of acid on k_3 was different from that on k_1 as hemicellulose is amorphous and cellulose is crystalline in nature. Furthermore, cellulose hydrolysis strongly depends on its degree of crystallinity and swelling state (Xiang et al., 2003). Using Eq. (5.4) and glucose formation model, it is possible to predict glucose concentration at each experimental point. The generalized model predicted maximum glucose level of 8.85 g/L at the most drastic conditions of acid concentration and residence time (6% H_2SO_4 and 120 min) but decreasing the residence time to <40 min, more than 1.89 g/L could be achieved for any acid concentration studied. The influence of H_2SO_4 concentration and residence time on the generalized model for predicting glucose concentration by response surface is shown in Figure 5.7. The graph illustrated that there was no degradation reaction leading to hydroxymethylfurfural (HMF) during hydrolysis. In order to extract maximum xylose, it is important to keep glucose concentration as minimum as possible in the hydrolysate. The response surface can help to select operating conditions that ensure the maximum xylose and minimum glucose yield.

A generalized model was developed to predict furfural concentration at any experimental conditions. The kinetic parameter k_4 was correlated with C_a by the empirical Eq. (5.5), and the determination coefficient R^2 was well fitted (Table 5.2). The surface graph for furfural generated in the hemicellulosic hydrolysate is shown in Figure 5.8. The figure showed that the model predicted a maximum furfural concentration of 1.81 g/L at the most severe conditions (6% H_2SO_4 and 120 min). The response surface depicted that furfural concentration continuously increased with increase in H_2SO_4 concentration and residence time. Furfural is an inhibitor of microbial fermentation (Arvela et al., 2011) and hence its level in the hydrolysate should be minimized in order to maximize xylitol production. The response surface suggested that a high acid

concentration and low residence time is favorable for maximum xylose and minimum furfural concentration in the hydrolysate.

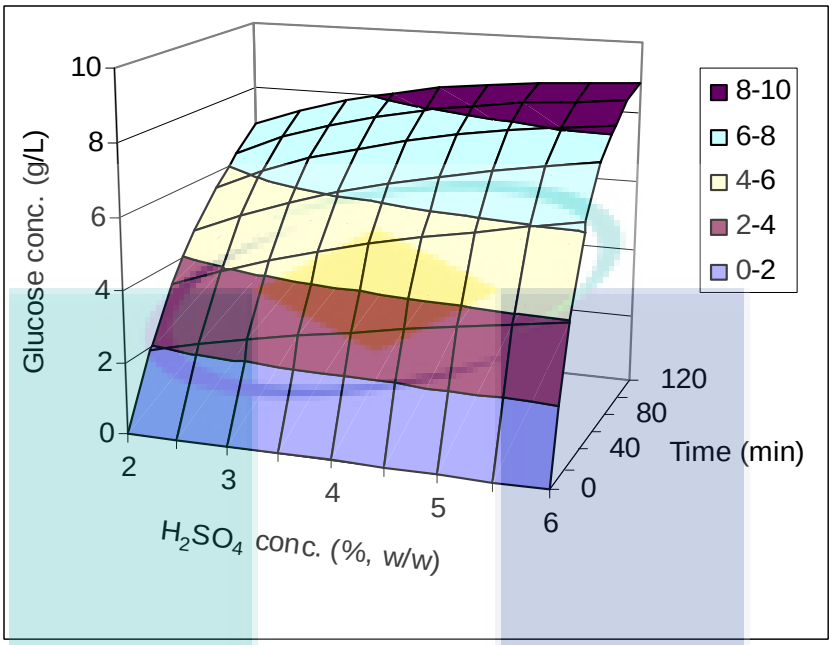


Figure 5.7: Influence of H_2SO_4 concentration and residence time on generalized model constructed for predicting glucose concentration

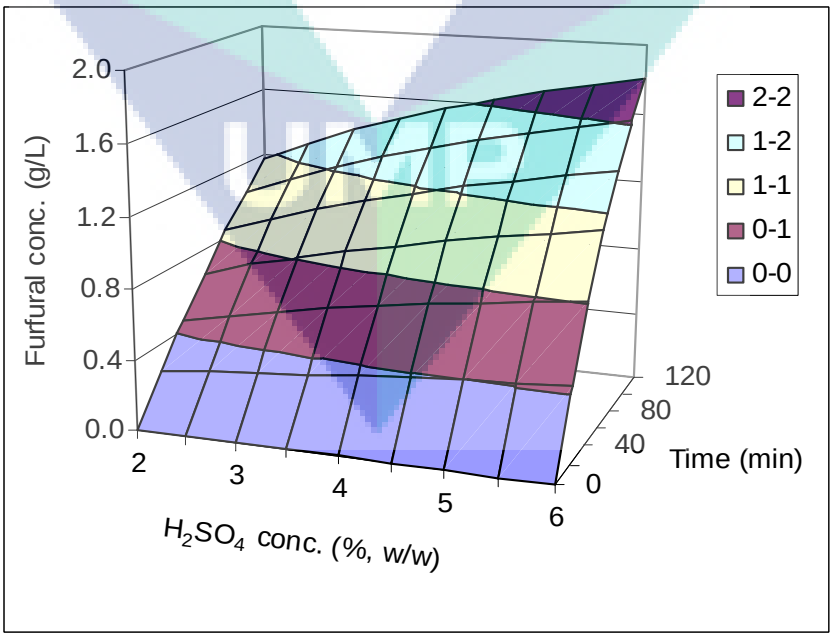


Figure 5.8: Influence of H_2SO_4 concentration and residence time on generalized model constructed for predicting furfural concentration

A generalized model was also developed in order to correlate k_5 with C_a for predicting acetic acid concentration for any residence time and acid concentration. This is represented by the empirical Eq. (5.6), and the determination coefficient R^2 fitted well (Table 5.2). The model predicted that the highest acetic acid concentration of 5.1 g/L is obtainable under the most severe conditions. The value of m for k_5 was 0.5411, which was lower than the corresponding value of m (1.2453) for k_1. The influence of H_2SO_4 on acetyl group removal from hemicellulose is lower compared to that on xylan yield, which may be interpreted as the reason for achieving distinct m values (Harris et al., 1985). The surface plot for predicting acetic acid formation with increase in sulfuric acid concentration and residence time is shown in Figure 5.9. The figure demonstrated that acetic acid concentration increased with increase in H_2SO_4 concentration and time. Therefore, it is better to perform the hydrolysis with high H_2SO_4 concentration and shorter residence time so that the acetic acid concentration can be kept at a minimum level.

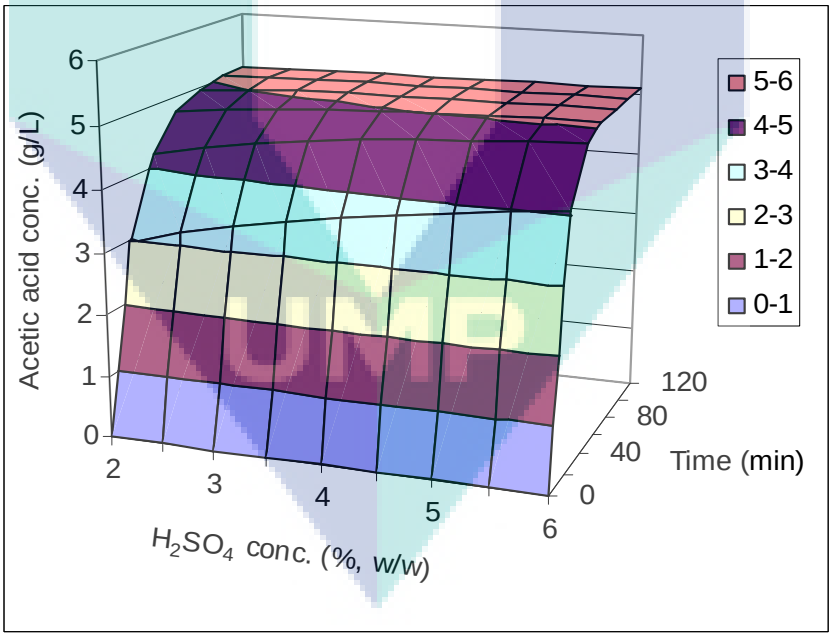


Figure 5.9: Influence of H_2SO_4 concentration and residence time on generalized model constructed for predicting acetic acid concentration

The generalized models for the hydrolysis of MWS recommended several options for achieving maximum xylose concentration. Using 2% H_2SO_4 for 60 min residence, the model predicted 16.64, 4.59, 0.66, and 4.73 g/L of xylose, glucose,

furfural, and acetic acid, respectively. Whereas using 4% acid for 40 min the predicted concentration of xylose, glucose, furfural and acetic acid were 17.98, 5.1, 0.66, and 4.7 g/L, respectively. On the other hand, 6% acid for 20 min residence time recommended 19.02, 3.99, 0.44, and 4.05 g/L of xylose, glucose, furfural, and acetic acid, respectively. The third recommended optimum parameters were tested in the laboratory and the concentrations of xylose, glucose, furfural, and acetic acid obtained were very close to the predicted values of 18.5, 4.2, 0.4, and 4.1 g/L, respectively. This behavior represented that the model adapted to the experimental results. The percentage errors were also well within acceptable value (5%), suggesting that the model adequacy to be reasonably accurate and reliable. Therefore, the optimum experimental conditions (obtained by kinetic study) for xylose recovery from MWS are 130 ºC, 6% H_2SO_4, and 20 min under which all the byproducts can be maintained to low level. Under these conditions, the yield was greater than 89% of the potential xylose concentration with a small concentration of inhibitory compounds (furfural and acetic acid) and negligible degradation of the cellulose fraction. Thus, the obtained kinetic models proved to be useful for further technical and economic studies.

During hydrolysis of MWS, xylose was obtained as the main product and at the same time, other undesired products such as glucose (4.21 g/L), arabinose (1.06 g/L), acetic acid (4.1 g/L), furfural (0.42 g/L), HMF (0.04 g/L), and LDPs (0.69 g/L) were also produced with the selectivity (defined as the ratio of xylose and undesired products) of 4.4, 17.5, 4.5, 44.1, 463.3, and 26.9 g/g, respectively, under the selected optimum conditions.

5.2.3 Optimization of Xylose Recovery by RSM

In the final step of optimization process, the central composite design (CCD) under response surface methodology (RSM) was used to develop quadratic model in order to obtain the true optimum conditions for maximum xylose yield and selectivity and to validate the CCD developed model. A careful optimization of process variables used for hydrolysis of MWS is important to ensure maximum xylose recovery and to minimize cogeneration of undesired products. The RSM is an efficient and popular experimental strategy to determine the optimum conditions for a multivariable system

rather than optimizing by the classical method. The CCD matrix with coded and actual variables, including experimental responses Y_1 (%) and Y_2 (g/g) is shown in Table 5.3. In this design, Y_1 (xylose yield) was defined by $[(X_c/X_p) \times 100]$, where X_c is the xylose concentration obtained in liquor by HPLC (g/L) and X_p is the potential concentration of xylose calculated (g/L) and Y_2 (selectivity), defined by xylose to glucose ratio. The results of response surface study on xylose recovery from MWS including model diagnosis are detailed hereafter.

Table 5.3: Experimental matrix with coded and actual variables, and results of CCD for xylose recovery

Run	Coded variables			Actual variables			Responses	
	A	***B***	***C***	***T* (ºC)**	***AC* (%)**	***C* (min)**	**Y_1 (%)**	**Y_2 (g/g)**
1	1	1	1	127	5	80	58.84 ± 1.56	2.28 ± 0.03
2	0	0	0	125	4	60	92.05 ± 1.89	3.72 ± 0.08
3	1	1	-1	127	5	40	84.24 ± 1.33	3.48 ± 0.19
4	0	0	0	125	4	60	86.41 ± 2.22	3.82 ± 0.05
5	1	-1	1	127	3	80	63.08 ± 1.61	3.64 ± 0.24
6	0	0	0	125	4	60	87.81 ± 1.86	3.81 ± 0.22
7	0	0	0	125	4	60	86.27 ± 1.50	4.00 ± 0.08
8	0	-2	0	125	2	60	70.65 ± 1.61	4.38 ± 0.23
9	-2	0	0	121	4	60	76.96 ± 2.11	5.44 ± 0.37
10	0	0	2	125	4	100	63.04 ± 1.40	2.84 ± 0.11
11	-1	1	-1	123	5	40	78.31 ± 1.82	4.93 ± 0.28
12	0	0	-2	125	4	20	37.59 ± 1.53	4.67 ± 0.31
13	2	0	0	129	4	60	60.96 ± 1.54	2.93 ± 0.13
14	-1	-1	-1	123	3	40	41.64 ± 1.27	4.71 ± 0.09
15	1	-1	-1	127	3	40	54.27 ± 1.20	4.35 ± 0.20
16	-1	-1	1	123	3	80	87.18 ± 2.68	4.84 ± 0.27
17	0	0	0	125	4	60	83.04 ± 1.51	3.76 ± 0.18
18	0	2	0	125	6	60	78.99 ± 3.39	2.70 ± 0.16
19	-1	1	1	123	5	80	90.22 ± 3.94	3.75 ± 0.13
20	0	0	0	125	4	60	81.40 ± 1.26	3.71 ± 0.10

T = temperature (*A*), *AC* = acid concentration (*B*), *C* = residence time
Y_1 = xylose yield (%, w/w), Y_2 = selectivity (g/g)

Diagnosis of Model Properties for Xylose Recovery

The statistical properties of model can best be diagnosed by inspecting various diagnostic plots such as plot of normal probability, studentized residuals, outlier T, and Box-Cox. The most important diagnostic plot is the normal probability plot of the studentized residuals. The studentized residuals are defined as the residuals divided by the calculated standard deviation of that residual which measures the number of standard deviations separating the actual and predicted values. The normal probability plot (Figure 5.10) depicted nearly a straight line of studentized residuals distribution, which indicates that the errors are evenly distributed and thus support the adequacy of the least square fit and the figure also denoted that neither response transformation was needed nor there was any apparent problem with normality.

In Figure 5.11, the studentized residual versus predicted response plot is illustrated. Ideally the plot should be a random scatter, suggesting that the variance of actual observations is constant for all values of the response. Figure 5.11 revealed that the proposed models are distinctly adequate and reasonably free from any violation of the independence or constant variance assumption, as studentized residuals lie in the range between 3 and –3. This result also indicated that there is no need for transformation of the response variable (i.e., Box-Cox plot).

The plot of outlier T is shown in Figure 5.12. The outlier T represents a measure of how many standard deviations of the actual value deviates from the predicted value. Most of the outlier T values should lie in the interval of ± 3.50 and any observation with a outlier T outside of this interval is potentially unusual with respect to its observed response (Körbahti and Rauf, 2008 and Montgomery, 2001). From the figure it was observed that all the outlier T values fall well within the red lines set at ± 3.50, indicating the approximation of the fitted model to the response surface was fairly good with no data recording error.

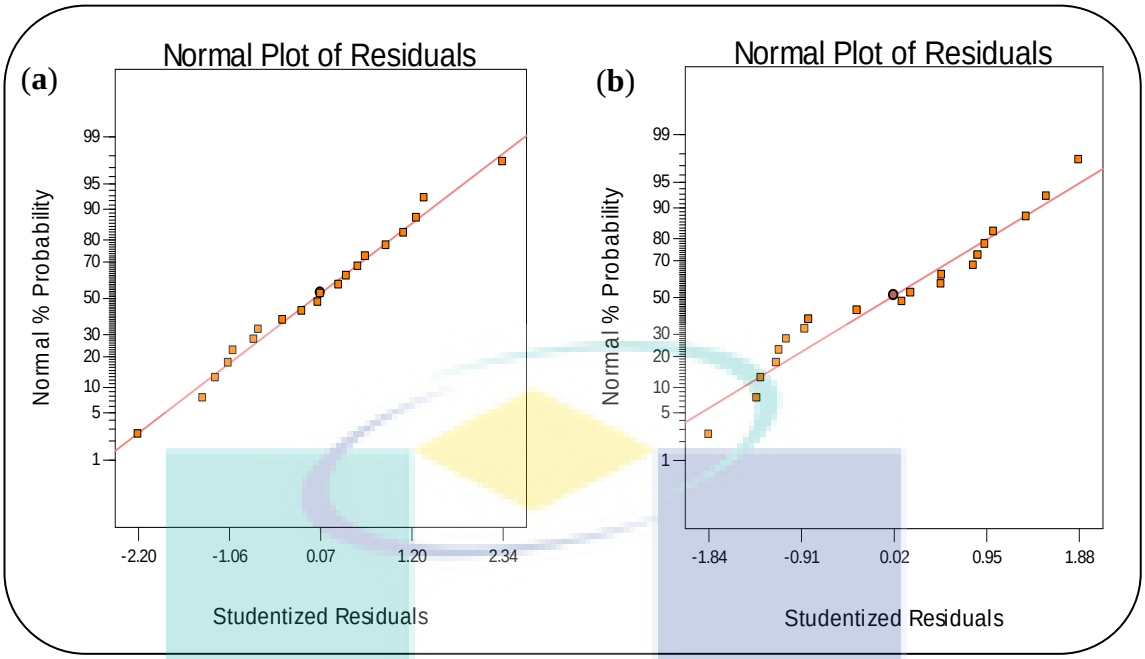


Figure 5.10: Normal probability plot of the studentized residuals for (a) xylose yield and (b) selectivity

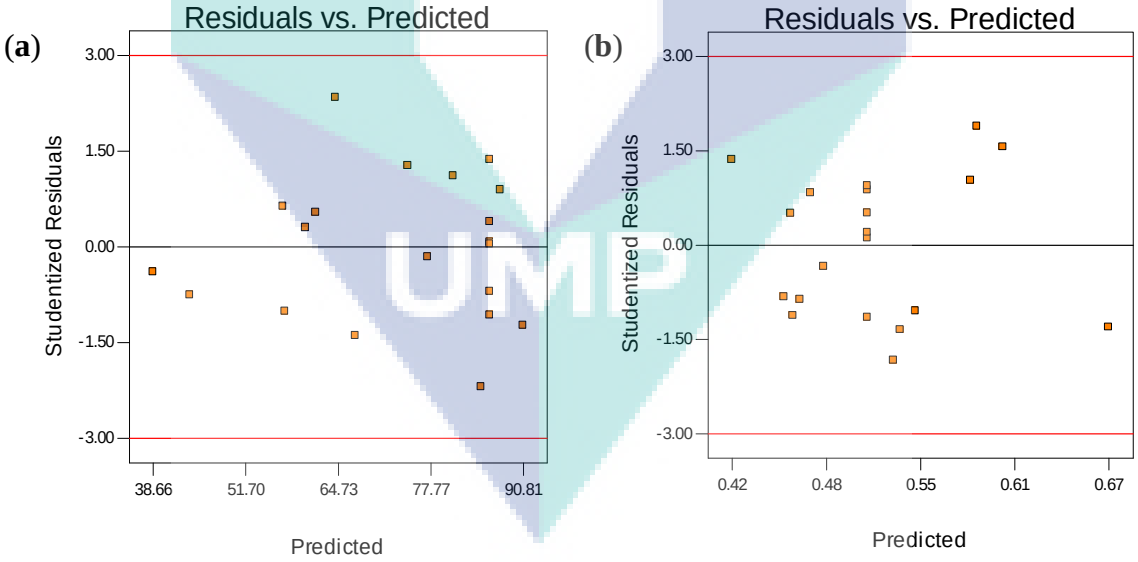


Figure 5.11: Plot of studentized residuals versus predicted responses: (a) xylose yield and (b) selectivity

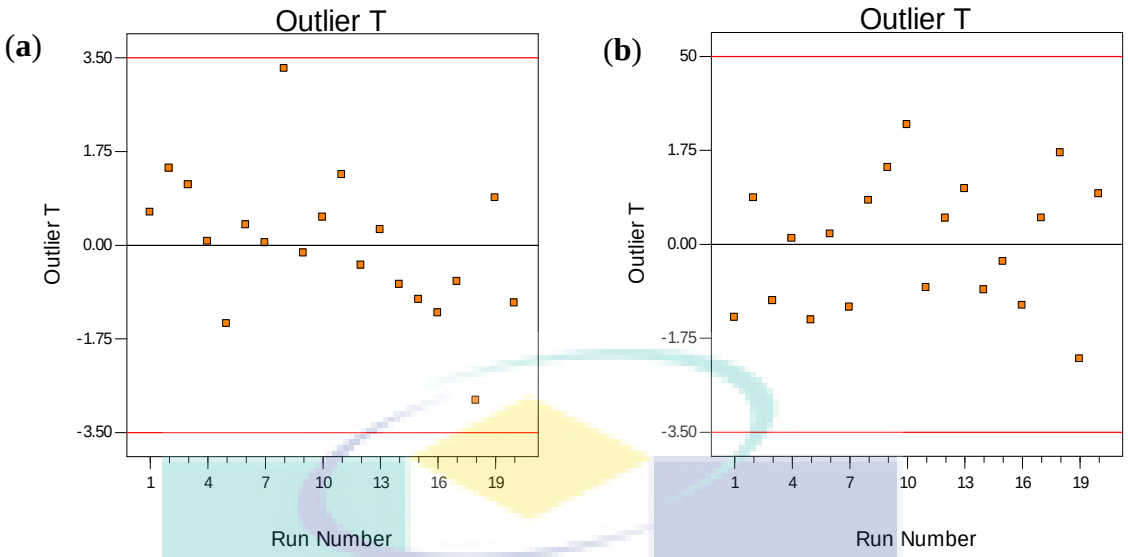


Figure 5.12: The outlier T plot for (a) xylose yield and (b) selectivity

Optimization of Process Condition

The effect of temperature (*A*), sulfuric acid concentration (*B*), and residence time (*C*) on the hydrolysis of MWS hemicellulose was evaluated with very little degradation of other fractions such as cellulose and lignin. The influence of these input variables was analyzed using CCD to determine the optimum conditions that will increase xylose yield and selectivity. The fit summary output analysis denoted that the quadratic models were statistically significant to represent both responses. The results of ANOVA for the quadratic model representing xylose yield and selectivity are presented in Table 5.4. The model adequacy was checked by *F*-test, *Prob* > *F*, determination coefficient (R^2) and correlation coefficient (*R*). As depicted in Table 5.4, the computed *F* and *Prob* > *F* value of model were 23.13 and <0.0001, respectively, indicating that the developed model was highly significant with low probability. These results adequately suggested that the obtained statistical model was in good prediction of the experimental results and the terms in the model have a significant effect on the response. Moreover, the R^2 for xylose yield was 0.9542, implying that 95.42% of the variability in the response could be well explained by the model while the remaining 4.58% of the total variation was elucidated by the residual. The correlation coefficient (*R*) value was calculated to be 0.9768, indicating a good agreement between the observed and predicted data of response. Furthermore, the lack of fit value was found insignificant (*Prob* > *F* = 0.1976) which proved that the obtained model was desirably fit.

Table 5.4: Analysis of variance (ANOVA) for the quadratic model representing xylose yield (Y_1) and selectivity (Y_2)

Source	Sum of squares		Degree of freedom		Mean square		*F*-value		*Prob > F*	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Model	4722.67	0.065	9	9	524.74	7.208E-003	23.13	72.60	< 0.0001[a]	< 0.0001[a]
A	296.87	0.025	1	1	296.87	0.025	13.09	250.95	0.0047	< 0.0001
B	421.48	0.016	1	1	421.48	0.016	18.58	162.73	0.0015	< 0.0001
C	526.24	0.015	1	1	526.24	0.015	23.20	152.53	0.0007	< 0.0001
A^2	471.82	1.816E-004	1	1	471.82	1.816E-004	20.80	1.83	0.0010	0.2061
B^2	206.66	1.061E-003	1	1	206.66	1.061E-003	9.11	10.69	0.0129	0.0084
C^2	2033.49	1.826E-004	1	1	2033.49	1.826E-004	89.64	1.84	< 0.0001	0.2049
AB	24.43	2.541E-003	1	1	24.43	2.541E-003	1.08	25.59	0.3238	0.0005
AC	685.24	1.530E-003	1	1	685.24	1.530E-003	30.21	15.41	0.0003	0.0028
BC	575.28	2.972E-003	1	1	575.28	2.972E-003	25.36	29.93	0.0005	0.0003
Residual	226.84	9.929E-004	10	10	22.68	9.929E-005				
Lack of fit	156.96	7.493E-004	5	5	31.39	1.499E-004	2.25	3.08	0.1976[b]	0.1215[b]
Pure error	69.88	2.436E-004	5	5	13.98	4.872E-005				
Cor total	4949.51	0.066	19	19						
R^2	0.9542	0.9849								
R	0.9768	0.9924								
Adj R^2	0.9129	0.9714								

Prob > F less than 0.050 indicate model terms are significant
[a] model is significant, [b] lack of fit is not significant

The quadratic effect of residence time (C^2) was found to be the most significant term to have the principal effect towards the xylose yield and this was followed by the interaction effect of temperature and residence time (*AC*), acid concentration and residence time (*BC*), and the main effect of residence time (*C*). Moreover, the quadratic effect of temperature (A^2), the main effect of acid concentration (*B*), temperature (*A*), and the quadratic effect of acid concentration (B^2) were found to be responsible for the secondary effect on the xylose yield. It should be noted that the interaction effect of temperature and acid concentration (*AB*) was found to be insignificant (*Prob* > *F* greater than 0.050 indicate model terms are insignificant). Hence, the ranking of model terms according to the statistical significance (based on the magnitude of *F*-value) in the study of xylose yield is as follows: $C^2 > AC > BC > C > A^2 > B > A > B^2$.

The model *F* and *Prob* > *F* value for selectivity were 72.6 and <0.0001 (Table 5.4), respectively, implying that the model was notably significant with low probability. The R^2 for selectivity was 0.9849, indicating that 98.49% of the variability in the response was well explained by the model while only 2.51% of the total variation was poorly described by the model. The *R* value was 0.9924, denoting a good agreement between the experimental and predicted values of selectivity. In addition, the model showed insignificant lack of fit (*Prob* > *F* = 0.1215) which suggested that the model representing selectivity was desirably fit. From the table it is remarked that the values of adjusted determination coefficients (adj R^2) for both xylose yield and selectivity were also very high (0.9129 and 0.9714, respectively), which indicate a high significance of models. The main effect of temperature (*A*), acid concentration (*B*), residence time (*C*), the interaction effect of acid concentration and residence time (*BC*), temperature and acid concentration (*AB*), temperature and residence time (*AC*), and squared effect of acid concentration (B^2) are significant model terms for selectivity. The quadratic effect of residence time (C^2) and temperature (A^2) seem to be insignificant to the proposed model. Thus, the ranking of significant model terms is $A > B > C > BC > AB > AC > B^2$.

The empirical models in terms of coded variables obtained as a function of temperature (*A*), acid concentration (*B*), and residence time (*C*), are shown in Eqs. (5.7) and (5.8), where Y_1 and Y_2 represents xylose yield and selectivity, respectively. To

minimize the error determination, the statistically insignificant terms were also included in the model equations.

$$Y_1 = +86.10 - 4.31A + 5.13B + 5.73C - 4.33A^2 - 2.87B^2 - 8.99C^2 - 1.75AB - 9.25AC - 8.84BC \quad (5.7)$$

$$Y_2 = +0.51 + 0.039A + 0.032B + 0.031C - 2.687\text{E-}003A^2 + 6.497\text{E-}003B^2 + 2.695\text{E-}003C^2 + 0.018AB + 0.014AC + 0.019BC \quad (5.8)$$

The obtained empirical equations are mathematical models that best described the correlation among the independent variables and the studied responses. Hence, these models can be used to predict and optimize the xylose production yield and selectivity within the experimental constraints.

Sugars and Byproducts Formation

The release of xylose, glucose, arabinose, and acetic acid in the hemicellulosic hydrolysate was dependent on the operational conditions examined, as shown in Figure 5.13a. The maximum and minimum xylose concentration of 19.1 and 7.8 g/L were obtained in run number 2 and 12 when experiments were conducted at 125 ºC with 4% H_2SO_4 and for 60 min and 20 min residence time, respectively (Figure 5.13a and Table 5.3). This indicated that the xylose concentration increased with the residence time. It was also observed that the xylose concentration decreased to 12.21 g/L in the experiment carried out at 127 ºC using 5% H_2SO_4 for 80 min (i.e., run 1), suggesting the degradation reactions to exist probably leading to furfural. Furthermore, the maximum level of arabinose, 2.88 g/L was achieved in run 19 by conducting the experiment at 123 ºC with 5% H_2SO_4 for 80 min (Figure 5.13a and Table 5.3). The arabinose concentration decreased to 2.11 g/L under the most severe condition in run 1. This result denoted that arabinose decomposed more rapidly than xylose. According to Carvalheiro et al. (2004b) arabinose exhibits a higher thermal susceptibility than xylose, and for this reason it is degraded first in the hemicellulosic hydrolysate. The CCD experimental run and concentration of various compounds released during H_2SO_4 hydrolysis of MWS are presented in Appendix B (Table B.5).

During acid hydrolysis, other sugars were also released to liquor mainly glucose, which can originate from cellulose, but principally from hemicellulose. However, glucose from cellulose is not usually produced at the same rate as that from hemicellulose under the operational conditions used for dilute acid hydrolysis (Téllez-Luis et al., 2002). It was noticed that the glucose concentration varied in each hydrolysate, and the highest and lowest amounts were obtained by run number 18 and 12, corresponding to 6.07 and 1.67 g/L, respectively (Figure 5.13a and Table 5.3). The low concentration of glucose and HMF (≤3.26 and ≤0.049 g/L, respectively) confirmed that the cellulosic fraction remained unaltered during acid treatment and thus the glucose obtained probably from the hemicellulose. Besides monomeric sugars, the resulting hemicellulosic hydrolysate also contained other byproducts such as acetic acid, furfural, HMF, and LDPs which act as inhibitors of microbial growth and xylitol production. Acetic acid is released by the hydrolysis of acetyl groups bound to the hemicellulosic sugars. In this study, the acetic acid concentration in the hydrolysate varied from 3.01 to 4.68 g/L (Figure 5.13a) while the highest concentration reached in run number 18 which was executed at 125 ºC using 6% H_2SO_4 and a residence time of 60 min (Figure 5.13a and Table 5.3). It was noticed that acetic acid generated in highest amounts during hydrolysis compared to other inhibitory byproducts in the hemicellulosic hydrolysate. It is reported that acetic acid concentration greater than 3 g/L acts as a potential inhibitor in the microbiological process of xylitol production by *Candida guilliermondii* using xylose-rich lignocellulosic hydrolysate (Felipe et al., 1995). The formation of furfural, HMF, and LDPs under several experimental conditions is illustrated in Figure 5.13b. Furfural is formed in small quantities as a degradation product from pentose sugars, ranging from 0.23 to 0.58 g/L and the maximum concentration attained in run 1 under the extreme condition (Figure 5.13b and Table 5.3). The low furfural concentration suggested a slight degradation of pentoses during hydrolysis. HMF generated from the degradation of hexoses was found in trace amounts in the hemicellulosic hydrolysate. The maximum HMF concentration of 0.12 g/L was also achieved in run 1 (Figure 5.13b and Table 5.3). These findings suggested that under severe conditions, pentoses were more susceptible to degradation than hexoses. Sugar decomposition can occur during hydrolysis if a high acid concentration is employed or when acid homogenization in the reactor is inadequate, creating regions with high acidity (Pessoa Jr et al., 1997).

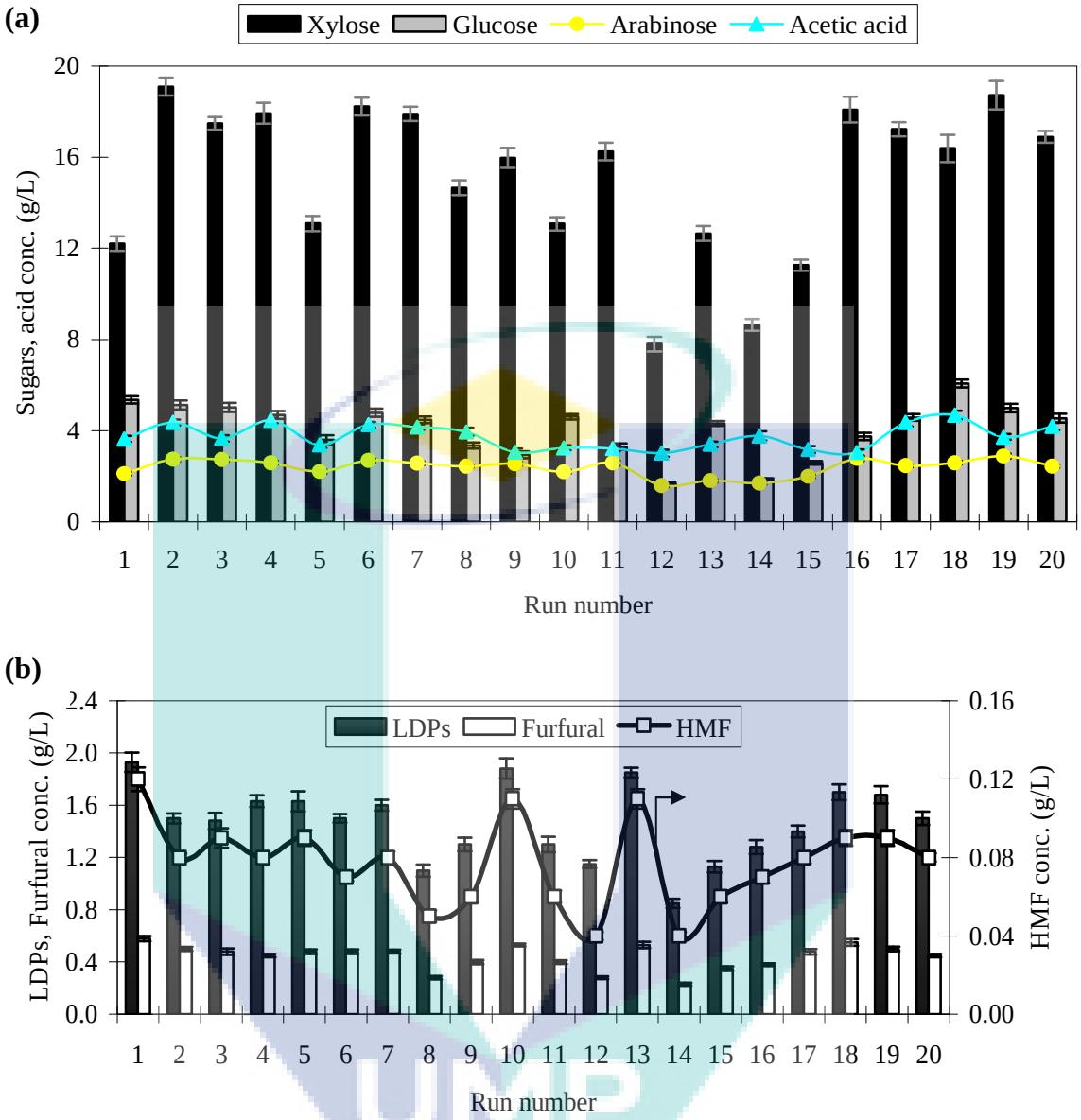


Figure 5.13: Under different hydrolysis conditions the formation of (a) sugars and acetic acid, and (b) sugar and lignin degradation products

Téllez-Luis et al. (2002) showed a continuous increase in furfural concentration during hydrolysis of sorghum straw when acid concentration and reaction time were increased. According to Neureiter et al. (2002) acid concentration is the most important parameter affecting sugar yield whereas temperature is mainly responsible for the formation of sugar degradation products. The potential toxic byproducts normally found in the hydrolysate are LDPs derived from lignin decomposition and extractive fraction. It was seen that LDPs concentrations in the hydrolysate vary from 0.85 to 1.93 g/L, which were obtained in run 14 and 1, respectively (Figure 5.13b and Table 5.3)

indicating the highest amount of LDPs produced under the drastic treatment condition. Parajó et al. (1998b) reported that LDPs are more toxic to xylose fermenting microorganisms than acetic acid, furfural, and HMF, even when found in low concentrations (<0.1 g/L). The concentration of furfural, HMF, and LDPs increased with increasing residence time, acid concentration and temperature. The reaction temperature slightly affects the decomposition reactions among the variables.

Interaction Effect of Variables

The interaction and response surface graphs generated to estimate the xylose yield and selectivity as a function of independent variables *A*, *B*, and *C* are shown in Figures 5.14, 5.15, and 5.16–5.18, respectively. The significance of interactions among the variables on the responses can be better understood using interaction graph. The effect of temperature and residence time on xylose yield when H_2SO_4 concentration was set at 4% as the centre point is shown in Figure 5.14(a, b). It was evident that the predicted xylose yield continuously deceased with the increase of temperature (from 92.07% at 123 ºC to 64.95% at 127 ºC), when residence time was maintained at 80 min. Conversely, an opposite result was found with the low level of residence time (40 min), in which the xylose yield increased from 62.09% at 123 ºC to 71.99% at 127 ºC (Figure 5.14(a, b)). These results demonstrated a negative correlation with temperature and positive correlation with residence time. In fact, heat could initially soften the lignin layer around the hemicellulose fiber which allows the acid to penetrate the layer and protonate the oxygen of glycosidic bond between monomeric sugars and subsequent breakdown of the bond leading to a rate enhancement. At higher temperature or longer residence time, the formed monosaccharides further breaks down to other compounds (such as furfural, HMF), resulting in the reduction of xylose yield. Canettieri et al. (2007) reported that a higher reaction temperature and time promoted the formation of sugar degradation products (e.g., furfural) and decreased the maximum xylose recovery attainable in the LCM hydrolysis process. Based on these results, it can be concluded that long residence time will give higher xylose yield (90.22%) as obtained by run 19 (Table 5.3). However, extremely long (100 min in run 10) or short (20 min in run 12) residence time decreased xylose yield to 63.04 and 37.59%, respectively.

The influence of H_2SO_4 concentration and residence time on xylose yield at medium level temperature (125 ºC), is shown in Figure 5.15(a, b). It was clear that the predicted xylose yield reduced with the increase of acid concentration (from 83.32% with 3% acid to 76.63% with 5% acid), when residence time was held at 80 min. A reciprocal result was found with the low residence time (40 min), in which the xylose yield linearly increased from 54.89% with 3% acid to 82.12% with 5% acid (Figure 5.15(a, b)). It can be concluded that low acid level will give higher xylose yield (87.18%) as demonstrated by run 16 (Table 5.3). At 6% acid concentration (in run 18), xylose yield decreased to 78.99%. However, using a relatively low acid concentration (2%) in run 8; xylose yield again reduced to 70.65%. From these results it is assumed that protons coming from low acid concentration can easily and effectively cleave the gylcosidic bonds in hemicellulose. At high acid concentration, the decomposition of monomeric sugars takes place simultaneously (xylose and arabinose are decomposed into furfural, and glucose into HMF) with the hydrolysis of polysaccharides. It is also assumed that long residence is required for protons diffusion in the wet lignocellulosic matrix and for diffusion of the reaction products into the liquid phase. As reported by Aguilar et al. (2002), a quantitative hydrolysis of the hemicelluloses can be done almost without damage to the cellulose because the bonds in hemicelluloses are weaker than in cellulose. Herrera et al. (2003) reported that acid hydrolysis of LCMs requires certain period of time for diffusion of protons from the bulk liquid phase to the wet lignocellulosic matrix and for reaction catalysis in order to hydrolyze the amorphous xylan to xylose.

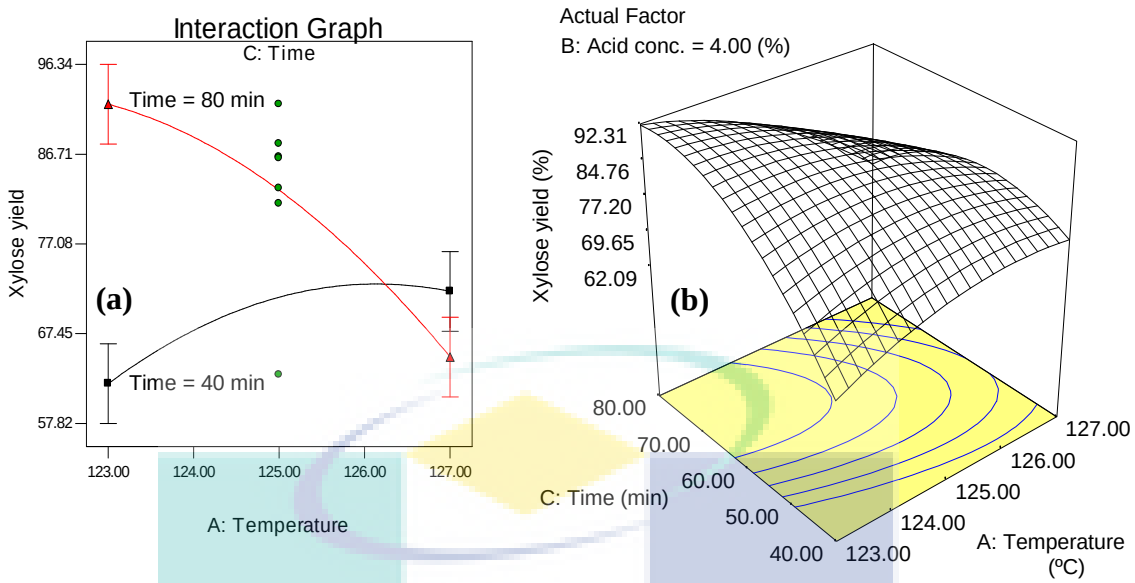


Figure 5.14: Effect of temperature and residence time on xylose yield: (a) interaction and (b) response surface graph

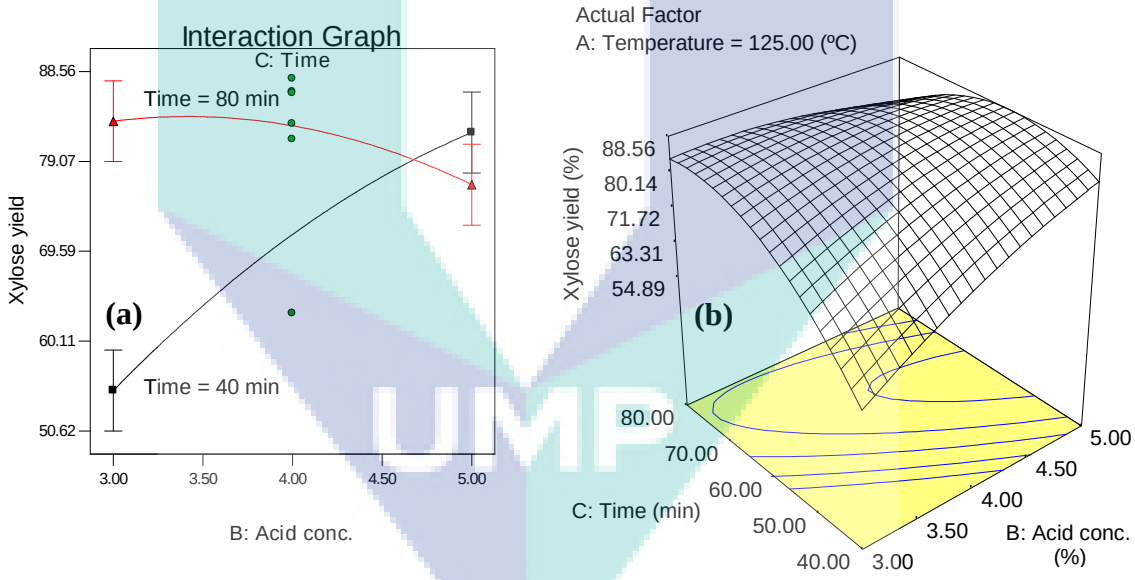


Figure 5.15: Effect of H_2SO_4 concentration and residence time on xylose yield: (a) interaction and (b) response surface graph

The effect of temperature and acid concentration on selectivity, keeping residence time at 60 min as the centre point is shown in Figure 5.16(a, b). The predicted selectivity increased at lower values for both temperature and acid concentration but decreased at higher values. The maximum selectivity of 4.7 g/g was predicted at the temperature and acid concentration of 123 ºC and 3%, respectively (Figure 5.16(a, b)). These results demonstrated that low temperature resulted in higher selectivity (4.71 g/g)

as achieved in run 14 (Table 5.3). At temperature 129 ºC (in run 13), selectivity dramatically decreased to 2.93 g/g. However, at a relatively low temperature (in run 9) selectivity increased to 5.44 g/g. The increase in selectivity attributed to the fact that low reaction temperature and acid concentration cause effective depolymerization of hemicellulose to yield maximum xylose while these conditions are not severe enough to hydrolyze cellulose resulting minimum glucose. Moreover, the selectivity diminished with increasing both temperature and acid concentration due to the degradation of xylose to furfural and/or the highest glucose recovery during MWS hydrolysis, which is consistent with the reported information (Parajó et al., 1994).

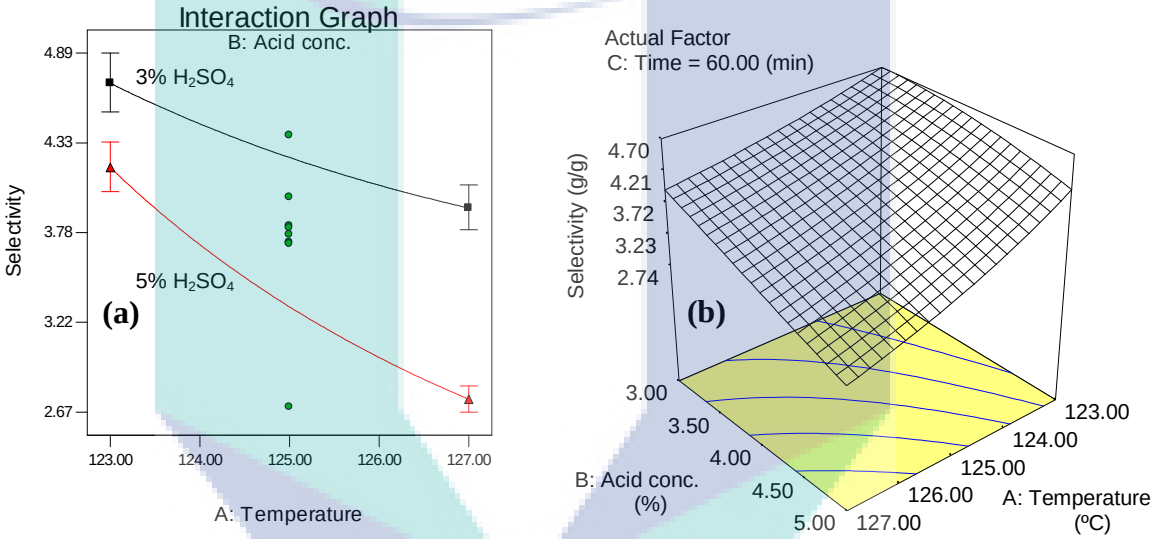


Figure 5.16: Effect of temperature and H_2SO_4 concentration on selectivity: (a) interaction and (b) response surface

The influence of temperature and residence time on selectivity, keeping H_2SO_4 concentration at 4% is shown in Figure 5.17(a, b). Selectivity decreased with increasing temperature and residence time. It was observed that the predicted selectivity declined with the increase of temperature (from 4.84 g/g at 123 ºC to 3.91 g/g at 127 ºC), while residence time was 40 min. Similar result was found with the high residence time (80 min), in which the selectivity decreased from 4.2 g/g at 123 ºC to 2.83 g/g at 127 ºC (Figure 5.17(a, b)). Based on these findings, it can be concluded that low residence time can give higher selectivity (4.71 g/g) as obtained by run 14 (Table 5.3). By maintaining the residence time at 100 min (in run 10), selectivity drastically decreased to 2.84 g/g. However, at a relatively low residence time (in run 12) selectivity increased to 4.67 g/g.

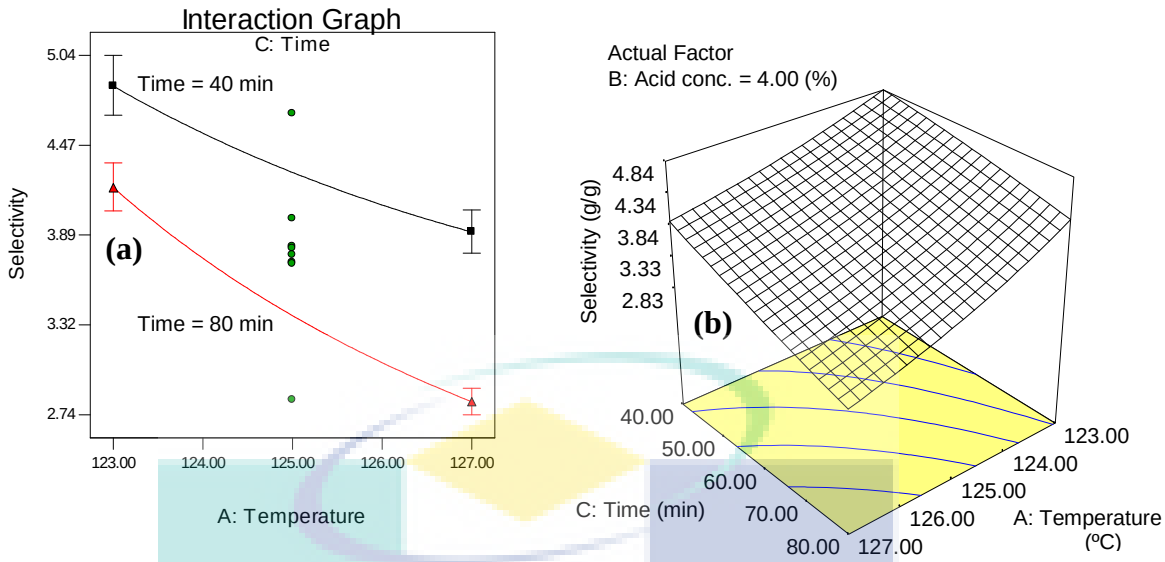


Figure 5.17: Effect of temperature and residence time on selectivity: (a) interaction and (b) response surface graph

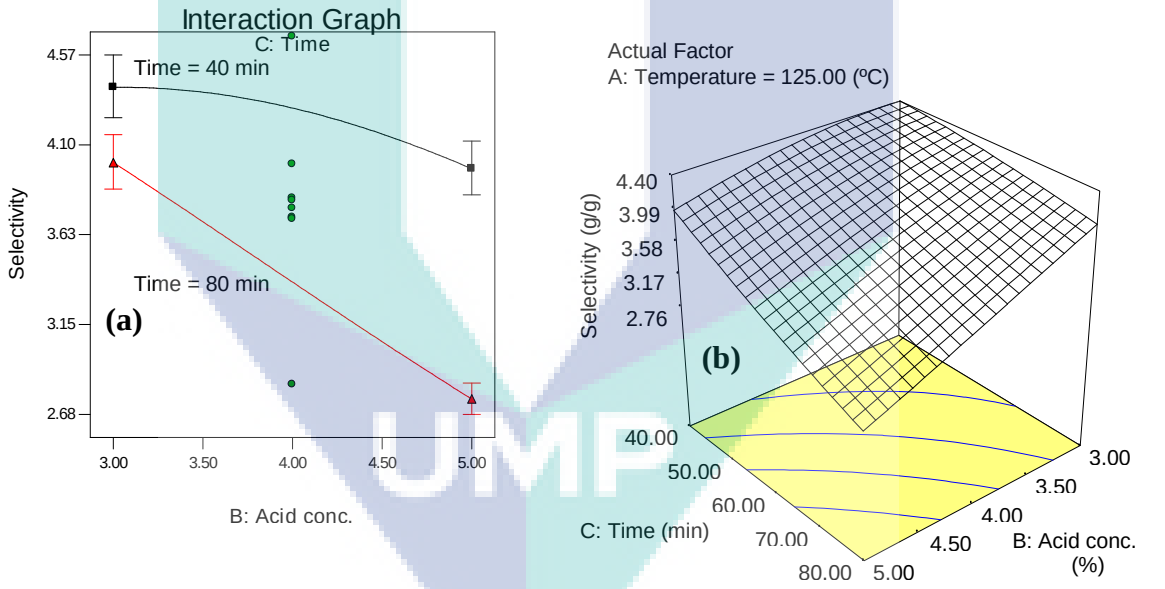


Figure 5.18: Effect of H_2SO_4 concentration and residence on selectivity: (a) interaction and (b) response surface graph

The effect of H_2SO_4 concentration and residence time on selectivity, at 125 ºC is shown in Figure 5.18(a, b). The predicted selectivity was enhanced at lower values of both acid concentration and residence time but diminished at higher values. The highest selectivity of 4.4 g/g was predicted at the temperature and residence time of 123 ºC and 40 min, respectively (Figure 5.18(a, b)). These results indicated that low acid concentration offers higher selectivity (4.71 g/g) as obtained in run 14 (Table 5.3).

Keeping acid concentration at 6% (in run 18) selectivity decreased to 2.7 g/g. However, further lowering of acid concentration (2%) in run 8; selectivity reached at 4.38 g/g.

Validation of Model Adequacy

Validation of the developed empirical model adequacy is necessary to justify the prediction accuracy. Based on both models, numerical optimization was executed with the 'Design Expert' program and five suggested optimal conditions were obtained, which are shown in Table 5.5. To verify these conditions, batch hydrolysis runs were conducted in triplicate under recommended optimum conditions. The acquired actual values and its associated predicted values from the verification runs were compared for residual and percentage error analysis. According to Eqs. (5.9) and (5.10) outlined by Zularisam et al. (2009), the error in percentage among the actual and predicted values of both responses over a considered ranges of operating variables were calculated.

$$\text{Residual} = (\text{Actual value} - \text{Predicted value}) \tag{5.9}$$

$$\%\,\text{Error} = \frac{\text{Residual}}{\text{Actual value}} \times 100 \tag{5.10}$$

The best results of xylose yield and selectivity obtained were 90.6% and 4.05 g/g, respectively when reaction temperature was 124 ºC, using acid concentration of 3.26% and residence time was 80 min that are typed in boldface in Table 5.5. It is evident that percentage errors ranged from 1.36 to 3.97% and 3.21 to 4.8% for xylose yield and selectivity, respectively. These findings indicated that the developed models were considerably adequate for both output variables as the percentage errors were well within acceptable value (5%), suggesting the model adequacy to be reasonably accurate within the 95% confidence interval (CI).

Table 5.5: Results of verification process with experimental design

Run variable			Xylose yield (%)				Selectivity (g/g)			
Temp (*A*)	**Conc. (*B*)**	**Time (*C*)**	**Actual**	**Predicted**	**Residual**	**Error (%)**	**Actual**	**Predicted**	**Residual**	**Error (%)**
124	3.25	80	89.52	91.83	-2.31	2.58	3.99	4.18	-0.19	4.76
124	**3.26**	**80**	**90.60**	**91.83**	**-1.23**	**1.36**	**4.05**	**4.18**	**-0.13**	**3.21**
124	3.28	80	88.67	91.83	-3.16	3.56	3.98	4.17	-0.19	4.77
124	3.31	80	88.31	91.82	-3.51	3.97	3.96	4.15	-0.19	4.80
124	3.24	80	89.40	91.67	-2.27	2.54	4.04	4.19	-0.15	3.71

Confirmation Experiment

Confirmation testing is an important and necessary step in the response surface method as it is a direct proof of the empirical model obtained. The results of confirmation run at the predicted optimum operating conditions are presented in Table 5.6. The optimum temperature, acid concentration and residence time for maximum xylose yield and selectivity were determined to be 124 ºC, 3.26% and 80 min, respectively. In these optimized conditions, the model predicted a maximum xylose yield and selectivity of 91.83% and 4.18 g/g, respectively, with a possible variation of 86.1 to 97.55% and 3.98 to 4.39 g/g, respectively, at 95% CI. To confirm these results, confirmation experiments were performed by employing the model suggested optimum conditions, and the xylose yield and selectivity obtained were 90.6% and 4.05 g/g, respectively. The optimum expected xylose yield and selectivity of 91.83% and 4.18 g/g respectively were very close to the confirmation results, indicating the models to be reasonably accurate and reliable. Hence, the obtained models could reliably be utilized for the prediction of optimum acid treatment conditions with respect to maximal recovery of xylose from wood sawdust.

Table 5.6: Results of confirmation run at the optimum operating conditions

Optimum conditions		**Xylose yield (%)**			**Selectivity (g/g)**		
		Actual	**Predicted**	**Error (%)**	**Actual**	**Predicted**	**Error (%)**
Temperature (ºC)	124						
Acid conc. (%, w/w)	3.26	90.60	91.83	1.36	4.05	4.18	3.21
Residence time (min)	80						

The xylose yield obtained by the statistical optimization technique (CCD) was 90.6%, a 5% improvement in the yield compared to that obtained by conventional OFAT approach (86.3%). Though low (5%), this improvement is important enough for the microbial growth and bioproduction of xylitol. The considered optimum conditions provided a hemicellulosic hydrolysate with an average initial pH of 0.66 and it contained 18.8 g/L xylose, 4.64 g/L glucose, 2.55 g/L arabinose, 4.14 g/L acetic acid, 0.55 g/L furfural, 0.08 g/L HMF, and 1.55 g/L LDPs. It is interesting to note that the hydrolysate contained low level of other components as glucose, arabinose, acetic acid, furfural, HMF, and LDPs, indicating an efficient hydrolysis. The reaction was shown to occur in the direction of the maximum breakdown of hemicellulose to xylose. Under optimized conditions, 91.84% of the hemicellulose was hydrolyzed. Thus, this condition was selected for subsequent generation of hemicellulosic hydrolysate in order to produce xylitol.

5.3 Conclusion

Hydrolysis of MWS was carried out in batch mode with sulfuric acid under selected experimental conditions to ensure high xylose recovery and to minimize byproducts in the resulting hemicellulosic hydrolysate. For optimizing xylose recovery, OFAT approach was firstly adopted in designing parameters and to primarily optimize the hydrolysis process. The maximum recovery of xylose was achieved when the reaction was conducted at 125 ºC for 60 min with 4% H_2SO_4 and a LSR of 8 g/g. The optimum concentration of xylose, glucose, arabinose, acetic acid, furfural, HMF and LDPs obtained were about 17.9, 4.7, 2.6, 4.5, 0.5, 0.1, and 1.5 g/L, respectively. It was found that xylose production was highly affected by temperature, acid concentration and residence time. The influence of these factors was further analyzed by RSM to determine the true optimum conditions in order to attain high xylose yield. Secondly, acid hydrolysis of MWS was accomplished at 130 ºC with several H_2SO_4 concentrations and residence time to study kinetics for xylose production. Hydrolysis reaction was assessed with the proposed kinetic models based on pseudohomogeneous irreversible first-order series reactions. The time course of the concentration of components in the hydrolysate was determined and the results were interpreted through the kinetic model which allowed a close reproduction of the experimental data. The maximum

concentration of xylose, glucose, furfural, and acetic acid achieved in the hydrolysate were 18.65, 8.81, 1.82, and 5.03 g/L, respectively. The parameters of kinetic models were obtained for predicting the concentration of products in the hydrolysate and used to optimize the process. Optimum H_2SO_4 concentration and residence time obtained at 130 ºC were 6% (w/w) and 20 min, respectively. The highest concentration of xylose, glucose, furfural, and acetic acid in the hydrolysate were 18.5, 4.2, 0.4, and 4.1 g/L, respectively. The obtained kinetic models proved to be useful for further technical and economic studies. Finally, a 2^3 full factorial CCD under RSM was employed to design experiments and to optimize the process with respect to xylose yield and selectivity. At optimum conditions, xylose yield and selectivity were 90.6% and 4.05 g/g, respectively, when temperature, acid concentration and residence time were 124 ºC, 3.26% and 80 min, respectively. It is mentioned that the CCD resulted in 90.6% xylose yield with a 5% increase as compared to that obtained with the classical OFAT method. In addition, about 92% of the hemicellulose was hydrolyzed in these conditions. It is worth noting that under controlled treatment conditions, MWS can be successfully used as a potential source of xylose. The main product xylose can be used as an economical carbon and energy source for the growth of microorganisms as well as a cheap starting raw material to bioproduction of xylitol, a high value product. Thus, the use of MWS to produce xylose will not only solve the disposal problem but also give back a high value product to the wood industry.

CHAPTER 6

SYNTHESIS OF XYLITOL FROM MWS HYDROLYSATE

The results of the studies on xylitol synthesis are presented in this chapter along with elaborate discussion. The outcomes of xylitol synthesis are presented in three steps namely, 'parameter design', 'screening', and 'optimization process'. The first step describes the use of OFAT method used to design various factors by observing their effects on xylitol production from xylose-rich MWS hemicellulosic hydrolysate (MWSHH) using XR. The second step describes the screening of significant variables influencing enzymatic xylitol production by fractional factorial design (FFD). Finally, the application of RSM for the optimization of xylitol synthesis is described. The observed results are discussed with respect to previously reported data. The performance of the novel enzymatic approach for xylitol production is also focused in this chapter.

6.1 INTRODUCTION

Xylitol is a natural pentahydroxy polyol with a similar sweetening power to sucrose (Parajó et al., 1998a and Winkelhausen and Kuzmanova, 1998). The global demand of xylitol is ever increasing because of its unique functional properties. Xylitol is industrially produced by catalytic reduction of pure D-xylose and can also be produced by biotechnological approaches. The chemical process is laborious, cost- and energy-intensive (Prakasham et al., 2009), and also poses environmental hazards as it utilizes a toxic Raney nickel catalyst and high pressure hydrogen gas. Biotechnological methods for xylitol production are based on the use of microorganisms or isolated enzymes. The microbial production of xylitol has been studied extensively in the past

few decades as an alternative to the chemical process (Ahmad et al., 2012; Barbosa et al., 1988; Horitsu et al., 1992; Jeon et al., 2011; Ko et al., 2006; Nigam and Singh, 1995 and Sampaio et al., 2008). In the microbial fermentation process using wild-type and recombinant yeast, the xylitol yield obtainable from xylose is in a range of 65–85% (Nigam and Singh, 1995) and 86–100% of the theoretical value (Bae et al., 2004 and Govinden et al., 2001), respectively. Despite the recombinant yeast's ability to bioconvert xylose to xylitol with almost theoretical yield, these strains could not produce xylitol for long periods due to an imbalance of the redox potential in the cell (Prakasham et al., 2009). The major advantage of the microbial process over chemical procedures is its lower cost due to the nonnecessity of extensive xylose purification (Parajó et al., 1998a). The application of the microbial process on an industrial level is time-consuming, being associated with some preparatory activities such as sterilization and routine inoculum development involving input of energy, labor, and time, leading to decreased productivity (Prakasham et al., 2009). Moreover, cell recycling in the microbial process requires membrane filtration, which makes it an unattractive method for the commercial manufacture of xylitol due to high membrane fouling problems (Granström et al., 2007a). However, the microbial process has not yet been able to accumulate the advantages of the chemical process because of the low productivity of xylitol and downstream processing problem.

Taking into account the bottleneck of microbial process, especially the necessity of huge sterile distilled water, long residence time and downstream processing problem, it is important to emphasize on the development of XR-dependent bioconversion of xylose from lignocellulosic hydrolysate. There are scarce reports regarding the enzymatic conversion of commercial pure xylose to xylitol by isolated XR from yeast (Kitpreechavanich et al., 1984; Nidetzky et al., 1996 and Neuhauser et al., 1998). Unfortunately, till now, no attempt is made for the *in vitro* enzyme-based production of xylitol from hemicellulosic hydrolysate by XR. The high cost involved in large-scale production of xylitol seems responsible for its limited commercial use. This has inspired the author to work on the development of improved technologies to lower the production costs. The enzymatic approach to xylitol production from xylose present in the LCMs may provide an alternative for the chemical process, and is safe and environment friendly. The hemicellulosic fraction of MWS biomass is easily

hydrolyzed by dilute acid to produce xylose-rich hydrolysate that can be used as potential substrate for enzymatic conversion to a variety of value-added products such as xylitol. Pure XR may offer an economic interest over the chemical and microbial production of xylitol from xylose present in the hemicellulosic hydrolysate. In this study, XR was produced in the laboratory from yeast *Candida tropicalis* IFO 0618 because of the high enzyme activity obtained from this organism (Horitsu et al., 1992 and Yokoyama et al., 1995).

It was reported that the reduction of xylose to xylitol by XR depended on the type and concentration of substrate, and experimental operating conditions. The reaction pH was considered to be a potential parameter that affects the ionization of functional groups on the enzyme involved in substrate binding and catalysis (Nidetzky et al., 1996, 2003). Temperature is found to be an important parameter influencing xylitol yield because at higher temperature, the XR inactivates rapidly, and at lower temperature, the reaction rate declines with temperature following the Arrhenius equation (Woodyer et al., 2005). Nidetzky et al. (1996) reported that a complete substrate bioconversion could not be achieved when more concentrated xylose solution (0.6–2.0 M) was employed in the production system. For a bioprocess, it is, therefore, necessary and important to optimize the process parameters that significantly influence the product yield.

The goal of the present work was to evaluate the effects of variables on xylitol synthesis from MWS hemicellulosic hydrolysate (MWSHH) and to optimize the process conditions. In order to obtain the optimum conditions for xylitol synthesis, this study was carried out in three steps. Firstly, the OFAT approach was applied to select the effective range of the factors. Secondly, the FFD was employed to define the most significant variables among reaction time, temperature, pH, NADPH concentration, and enzyme concentration, which affect xylitol yield. Finally, the response surface methodology (RSM) was followed to establish the true optimum conditions for the improvement of xylitol yield and productivity.

6.2 RESULTS AND DISCUSSION

6.2.1 Parameter Designing by OFAT for Xylitol Synthesis

The purpose of the OFAT study was to investigate the factors that impact the enzymatic process, namely reaction time, temperature, pH, xylose and NADPH concentration, enzyme concentration, and agitation rate on xylitol biosynthesis from MWSHH and to design parameters for further optimization studies. OFAT is a simple experimental strategy where the individual effects of factors on the response can be observed on graphs (Kumar et al., 2003 and Panda et al., 2007). In this study, OFAT method was used to determine the possible optimum levels of factors for xylitol synthesis due to the lack of information on the *in vitro* production of xylitol from lignocellulosic substrate using XR. The effects of these seven factors on xylitol synthesis, examined with OFAT, are described in the following subsections.

Effect of Reaction Time

Enzymatic conversion was carried out at various levels of reaction time varying from 2–18 h for maximizing xylitol production (Table 3.3). The influence of reaction time on xylitol production at fixed temperature 25 °C, pH 6.0, xylose concentration 18.8 g/L, NADPH concentration 2.83 g/L, enzyme concentration 5% (v/v), and agitation 150 rpm is shown in Figure 6.1. Xylitol yield gradually increased with reaction time up to 10 h with a maximum value of 41.12% (w/w) corresponding to 7.73 g/L of xylitol (Figure 6.1). However, further increase in reaction time did not increase xylitol yield at all. The linearity in xylitol yield during reaction time above 10 h might be due to coenzyme (NADPH) depletion or enzyme inactivation. A duration of 8–12 h reaction was suitably chosen for further study by FFD for screening the significant variables to achieve the highest xylitol yield. A rough optimum reaction time was assumed to be 10 h for further experiments to study the effect of temperature, pH, xylose concentration, NADPH concentration, enzyme concentration, and agitation rate on xylitol synthesis. The OFAT design matrix and the experimental data of xylitol production from MWS hydrolysate using XR are presented in Appendix B (Table B.6).

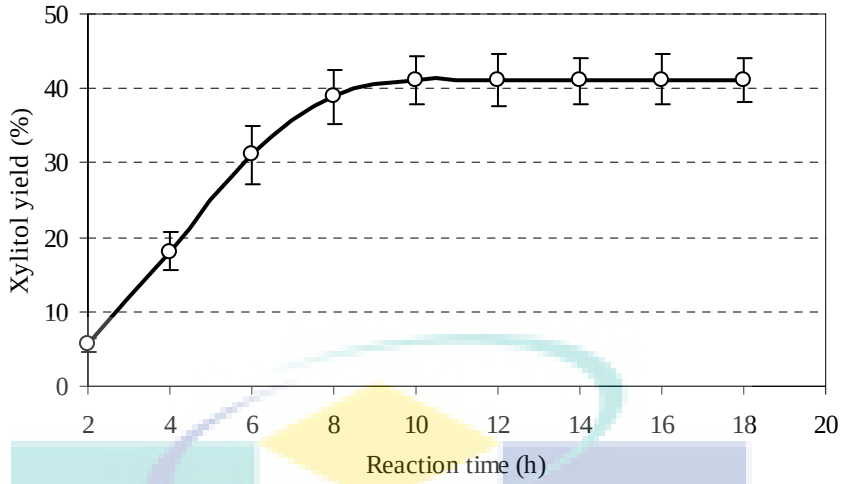


Figure 6.1: Effect of reaction time on xylitol production at 25 °C, pH 6.0, xylose 18.8 g/L, NADPH 2.83 g/L, XR enzyme 5%, and agitation 150 rpm

Effect of Temperature

A set of temperatures ranging from 20–70 ºC were applied to study the influence of temperature on xylitol biosynthesis. As shown in Figure 6.2, the yield of xylitol increased with increase in temperature and gave the highest value of 51.17% (w/w) at 30 ºC while other factors were kept constant (Table 3.3) and the yield remained constant up to 40 ºC. The yield decreased rapidly on further increase of temperature, and at 60 ºC, it reached to 15.32% that corresponds to a 3.3-fold reduction in the highest value. This decrease in xylitol yield at temperatures above 40 ºC is due to a progressive loss in XR activity or inactivation of NADPH (NADPH remained active up to 40 ºC) according to the subsection 4.2.2, effect of temperature on XR activity and stability. This result is consistent with the report that XR enzyme is almost completely inactivated after 1 h at 60 ºC (Yokoyama et al., 1995). A significant amount of xylitol was obtained within temperatures 25–35 ºC and thus the possible optimum temperature was chosen to be at 30 ºC for subsequent experiments.

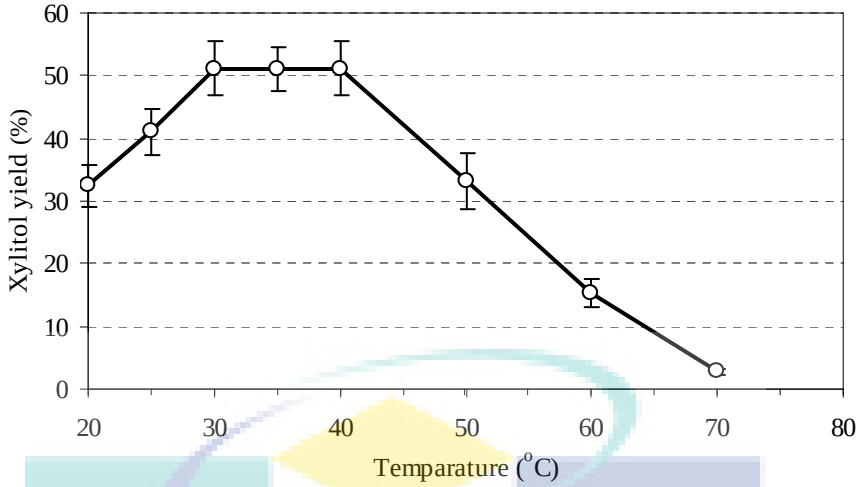


Figure 6.2: Effect of temperature on xylitol production at pH 6.0, reaction time 10 h, xylose 18.8 g/L, NADPH 2.83 g/L, XR enzyme 5%, and agitation 150 rpm

Effect of pH

To find out a rough optimum pH, different pH values ranging from 4.0–9.0 (different buffers described in subsection 3.7.8) were employed. Figure 6.3 presents the effect of pH on xylitol production at 30 ºC, 10 h reaction time, 18.8 g/L xylose, 2.83 g/L NADPH, 5% (v/v) enzyme, and 150 rpm agitation rate. The highest yield of xylitol was 51.65% (w/w) at pH values 6.0–7.0 (Figure 6.3). The initial reaction pH is a critical factor for NADPH-dependent reduction of xylose by XR, which affects the ionization of the functional groups involved in substrate binding and catalysis (Nidetzky et al., 2003). At alkaline condition (pH >7.0), the yield of xylitol sharply decreased probably due to the oxidation of xylitol to xylulose. Lee et al. (2003) reported that the requirement of an acidic pH for xylose reduction and an alkaline pH for xylitol oxidation are general features of similar XRs isolated from diverse organisms. The results demonstrated in Figures 4.3 and 4.4 also confirmed the stability test done earlier (subsection 4.2.2 of chapter 4) that pH 7.0 is favorable for the catalytic activity of XR. Thus, an initial pH value of 7.0 was selected for further experiments to examine the influences of the rest of process factors.

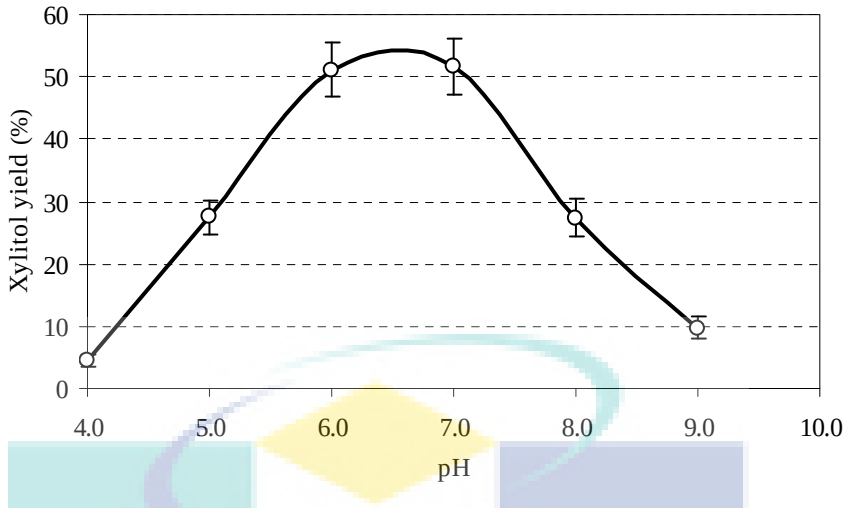


Figure 6.3: Effect of pH on xylitol production at 30 °C, reaction time 10 h, xylose 18.8 g/L, NADPH 2.83 g/L, XR enzyme 5%, and agitation 150 rpm

Effect of Xylose Concentration

Xylose concentrations in the reaction mixture was maintained at 7 different levels ranging from 9.4–37.6 g/L by either diluting or concentrating the MWSHH, to evaluate its influence on the formation of xylitol and is depicted in Figure 6.4. It was observed that a maximum xylitol yield of 51.65% was obtained with 18.8 g/L xylose, and thereafter gradually declined probably due to the increased amounts of inhibitory compounds (such as furfural, HMF, LDPs) compared to the non-concentrated MWSHH. It is reported that the initial xylose concentration is typically in a range of 37.5–75 g/L (0.25–0.5 M) with 96% conversion of pure D-xylose to xylitol whereas, in case of more concentrated xylose solution (0.6–2.0 M), a complete substrate conversion could not be obtained (Nidetzky et al., 1996 and Neuhauser et al., 1998). An appreciable amount of xylitol was produced from the crude and non-concentrated MWSHH containing 18.8 g/L xylose (Figure 6.4). Thus, xylose concentration was maintained at 18.8 g/L in the subsequent experiments on xylitol production in order to avoid the inhibitory effect of byproducts. The initial xylose concentration is also a critical factor in xylitol production. In the enzyme-based bioconversion, a high initial xylose concentration results in a high product yield and is more economically viable in terms of product recovery, but with the drawback of retarded enzyme activity. Nidetzky et al. (1996) pointed out that the produced xylitol is another factor, which may exhibit an inhibitory effect (i.e., product inhibition) on the XR. Therefore, the effects of inhibitors on xylitol production await further study.

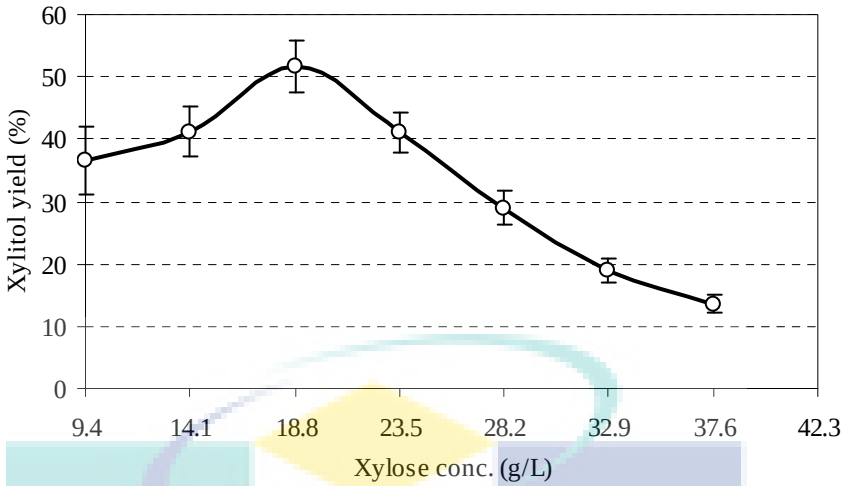


Figure 6.4: Effect of xylose concentration on xylitol production at 30 °C, pH 7.0, reaction time 10 h, NADPH 2.83 g/L, XR enzyme 5%, and agitation 150 rpm

Effect of NADPH Concentration

The effect of coenzyme NADPH concentration on the production of xylitol was studied within the ranges of 1.17–5.32 g/L. It was observed that xylitol yield continuously increased with the increase of NADPH concentration up to 3.66 g/L with a maximum value of 53.83%. The yield of xylitol remained almost unchanged with further increase of NADPH (Figure 6.5). The presence of NADPH above the saturating concentration (i.e., supersaturation) could slow the conversion of xylose to xylitol. A similar result has been drawn from characterization studies of purified XR (Yokoyama et al., 1995). Figure 6.5 presents the effect of NADPH concentration on xylitol production. It was also seen that a considerable amount of xylitol was produced from 2.0–3.66 g/L NADPH that was further studied by FFD for screening the most significant factors. The observed optimum NADPH concentration was 3.66 g/L that was used in the subsequent experiments.

Effect of Enzyme Concentration

The impact of xylose reductase (XR) on the synthesis of xylitol was investigated in the concentration range of 2–6% (v/v) (0.22–0.66 U/mL of reaction volume). The effect of enzyme concentration on xylitol production keeping other factors constant (Table 3.3) is shown in Figure 6.6. The low concentration of XR (2%) gave moderate

yield of xylitol (53.83%), but as the concentration increased to 3% the yield increased to 56.01% and then remained constant up to 4%. It was observed that the yield decreased with further increase in enzyme concentration (>4%) (Figure 6.6). At higher XR concentration compared to substrate, all enzymes could not combine with substrates, which might result in a remarkable reduction in the corresponding product. These results indicated that the production of xylitol might not be favorable with the high concentration of XR. Seelbach and Kragl (1997) studied the enzymatic synthesis of leucine from trimethylpyruvate using leucine dehydrogenase (LeuDH) and formate dehydrogenase (FDH) with the regeneration of coenzyme (NADH), and reported that the loading of excessive amount of enzyme to the reaction medium resulted in the decrease in leucine formation. The probable optimum enzyme concentration was found to be 3% (0.33 U/mL) that was employed in the subsequent experiments. The effect of enzyme concentration was further statistically calculated in the range of 2–4% using FFD.

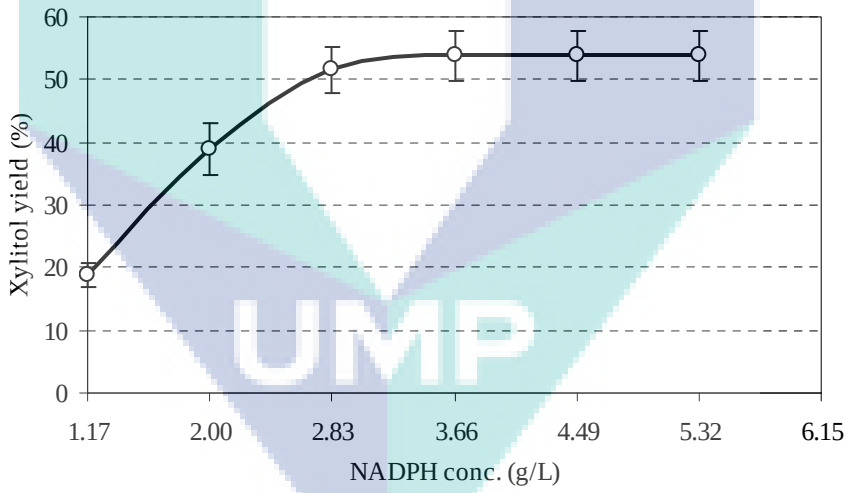


Figure 6.5: Effect of NADPH concentration on xylitol production at 30 °C, pH 7.0, reaction time 10 h, xylose 18.8 g/L, XR enzyme 5%, and agitation 150 rpm

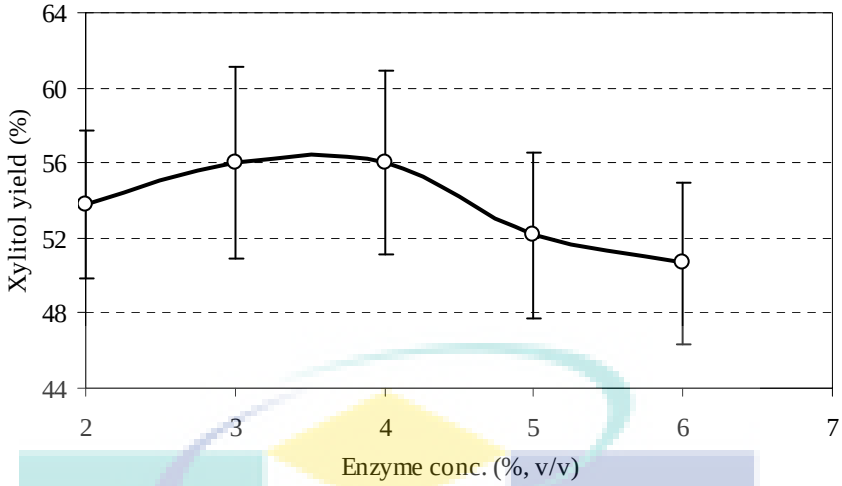


Figure 6.6: Effect of XR enzyme concentration on xylitol production at 30 °C, pH 7.0, reaction time 10 h, xylose 18.8 g/L, NADPH 3.66 g/L, and agitation 150 rpm

Effect of Agitation Rate

Agitation rate was varied between 50 and 150 rpm in order to monitor its influence on the production of xylitol. Figure 6.7 demonstrates the effect of agitation rate on xylitol production. It was noticed that the variation of agitation rate to a certain level showed an appreciable improvement in xylitol production (Figure 6.7). A medium agitation rate of 100 rpm supported maximum xylitol yield with a value of 56.01%. The yield of xylitol did not increase with further increase of agitation rate. The influence of agitation on xylitol production was less significant possibly due to the homogeneity of the reaction or to the limited oxygen requirement. A number of studies have reported that oxygen-limited conditions favored xylitol synthesis from xylose by NADH-linked XR in yeast cells because of NADH accumulation that subsequently inhibits the NAD-linked XDH (Branco et al., 2009; Parajó et al., 1998a; Winkelhausen and Kuzmanova, 1998 and Zhang et al., 2012). The agitation parameter was set at 100 rpm throughout the studies as it did not show any positive effect on the production of xylitol at high level. In addition, lower the agitation rate is the lesser energy expenditure.

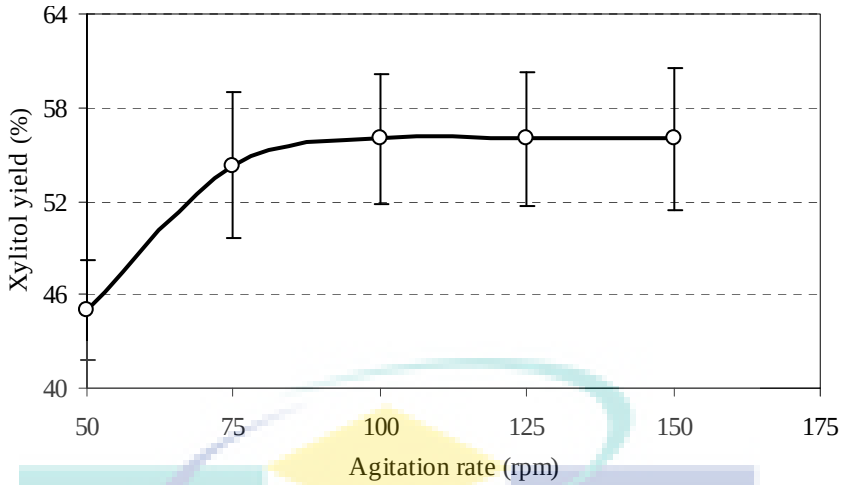


Figure 6.7: Effect of agitation on xylitol production at 30 °C, pH 7.0, reaction time 10 h, xylose 18.8 g/L, NADPH 3.66 g/L, and XR enzyme 3%

The observed optimum operating conditions determined by OFAT study for xylitol synthesis are: reaction time 10 h, temperature 30 ºC, pH 7.0, xylose concentration 18.8 g/L, NADPH concentration 3.66 g/L, enzyme concentration 3% (v/v) (0.33 U/mL), and agitation rate 100 rpm. The highest xylitol yield (56.01% or 10.53 g/L) was obtained under these conditions. The OFAT study revealed that among the seven factors examined, five factors (reaction time, temperature, pH, NADPH concentration, and enzyme concentration) markedly influenced the biosynthesis of xylitol from MWSHH. Other two factors such as xylose concentration and agitation rate were kept constant at 18.8 g/L and 100 rpm, respectively, throughout the investigations due to their insignificant effect on xylitol production and to avoid the inhibitory effect and reduce energy consumption. The experimental data were analyzed by linear regression using Minitab® software to confirm the OFAT results. Table 6.1 shows the ANOVA output for the effect of various factors studied by OFAT on xylitol synthesis. These results signified that reaction time, temperature, pH, NADPH concentration, and enzyme concentration imparted significant effect on the production of xylitol (as their *Prob* > *F* values are less that 0.050). Besides, these factors also revealed strong relationship with xylitol production and depicted the satisfactory correlation coefficient (*R*; often called Pearson correlation) values of 0.929, 0.879, 0.937, 0.802, and 0.900, respectively. The effect of other two factors (xylose concentration and agitation rate) was insignificant (the value of *Prob* > *F* above 0.050 indicate factors are insignificant) (Table 6.1). It is noted here that the significant factors were found consistent with the

graphical presentations of OFAT studies. Therefore, the effects of these five factors were studied further by FFD for identifying the most significant variables by taking into account their interaction effect. Hence, the single factor design proved to be important aiming at the search of the optimum range of variables in a bioconversion process.

Table 6.1: ANOVA for the effect of various factors studied by OFAT on xylitol synthesis ($\alpha = 0.050$)

Factors studied		**Description of regression**	**Value**	**Result**
Name	**Unit**			
Reaction time	(h)	Correlation coefficient	0.929	
		R^2 (%)	86.4	
		Type of regression model	Linear	Significant
		F-value	21.69	
		Probability > *F*	0.0001	
Temperature	(ºC)	Correlation coefficient	0.879	
		R^2 (%)	77.2	
		Type of regression model	Linear	Significant
		F-value	28.348	
		Probability > *F*	0.013	
pH		Correlation coefficient	0.937	
		R^2 (%)	87.8	
		Type of regression model	Linear	Significant
		F-value	5.49	
		Probability > *F*	0.002	
Xylose conc.	(g/L)	Correlation coefficient	0.776	
		R^2 (%)	60.3	
		Type of regression model	Linear	Insignificant
		F-value	2.34	
		Probability > *F*	0.079	
NADPH conc.	(g/L)	Correlation coefficient	0.802	
		R^2 (%)	64.3	
		Type of regression model	Linear	Significant
		F-value	9.52	
		Probability > *F*	0.002	
Enzyme conc.	(%, v/v)	Correlation coefficient	0.900	
		R^2 (%)	81.1	
		Type of regression model	Linear	Significant
		F-value	12.131	
		Probability > *F*	0.041	
Agitation rate	(rpm)	Correlation coefficient	0.797	
		R^2 (%)	63.5	
		Type of regression model	Linear	Insignificant
		F-value	1.88	
		Probability > *F*	0.110	

6.2.2 Screening of Variables by FFD for Xylitol Synthesis

The second step of seeking optimum conditions is to screen the input variables that have the greatest effect on the response, xylitol yield. Screening experimental designs are statistical techniques where many variables are selected concurrently to a significant few and much quantitative information can be acquired through a few numbers of experiments (Bouchekara et al., 2011 and Luo et al., 2009). These designs are needed to determine which of the variables and their interactions present more significant effects on the response and should be retained in subsequent models (Bezerra et al., 2008). In addition, a screening design is employed to reduce the number of parameters in order to decrease the required computational time and process cost (Bouchekara et al., 2011). Fractional factorial design (FFD) is one of the most powerful and widely used statistical screening approach for the identification of important variables affecting process response principally because it is efficient and economical (Bezerra et al., 2008 and Silva and Roberto, 1999). A more recent investigation pointed out that the FFD is successfully used to reduce both cost and time of a process where it aims to establish a design experiment with less number of tests (Bouchekara et al., 2011). FFD was followed in this study to identify the most important factors influencing xylitol synthesis among the variables involved. Five input variables namely reaction time (X_1), temperature (X_2), pH (X_3), NADPH concentration (X_4), and enzyme concentration (X_5) were taken into consideration according to the findings of single factor experiments (subsection 6.2.1) with xylitol yield ($Y_{p/s}$) as an output variable. The FFD layout and observed and predicted values of the screening process are listed in Table 6.2. The results of FFD study are presented in the following subsection.

Table 6.2: Experimental layout of 2^{5-1} FFD with xylitol yield ($Y_{p/s}$) values

Run	Coded variables					Xylitol yield ($Y_{p/s}$, %)	
	X_1 Time	X_2 Temp	X_3 pH	X_4 NADPH	X_5 Enzyme	Predicted	Experimental
1	0	0	0	0	0	73.25	73.83 ± 3.91
2	-1	-1	1	1	1	55.13	54.89 ± 1.51
3	0	0	0	0	0	73.25	72.66 ± 3.74
4	1	1	1	1	1	70.77	71.12 ± 2.92
5	1	-1	1	1	-1	58.97	58.62 ± 2.48
6	0	0	0	0	0	73.25	73.62 ± 3.76
7	-1	-1	-1	1	-1	58.44	58.68 ± 2.04
8	-1	1	1	-1	1	62.00	61.65 ± 2.81
9	1	1	-1	1	-1	68.59	68.24 ± 3.74
10	1	-1	-1	1	1	57.15	57.50 ± 1.86
11	1	-1	-1	-1	-1	67.42	67.18 ± 3.12
12	1	1	1	-1	-1	68.27	68.03 ± 4.46
13	-1	1	-1	1	1	55.61	55.37 ± 2.38
14	1	-1	1	-1	1	66.20	66.44 ± 2.95
15	0	0	0	0	0	73.25	73.30 ± 4.89
16	-1	1	1	1	-1	56.89	57.13 ± 2.48
17	0	0	0	0	0	73.25	71.54 ± 3.15
18	1	1	-1	-1	1	64.89	65.13 ± 4.53
19	0	0	0	0	0	73.25	74.57 ± 5.51
20	-1	1	-1	-1	-1	49.54	49.89 ± 1.48
21	-1	-1	-1	-1	1	50.03	49.68 ± 1.33
22	-1	-1	1	-1	-1	62.12	62.47 ± 3.89

Screening of Significant Variables by FFD

Experimental data were systematically analyzed via a 2^{5-1} fractional factorial design (FFD) as a screening approach by investigating the main and interaction effects of reaction time, temperature, pH, NADPH concentration, and enzyme concentration on xylitol yield, as summarized in Table 6.3. A statistical testing was carried out using *F*-test for ANOVA, which was applied to determine the significant factors where degree of significance was ordered according to the *F*-value. In fact, the larger the magnitude of *F*-value and the smaller the *Prob* > *F* value the more significant are the corresponding model and the individual coefficient (Montgomery, 2001). From the Table 6.3 it was found that the *F*- and *P*-values of the model were 50.44 and <0.0001, respectively, implying that the model constructed was significant as well as it fitted the experimental data adequately.

Table 6.3: ANOVA of the first order model fitted to xylitol yield ($Y_{p/s}$)

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*Prob* > *F*
Model	648.14	13	49.86	50.44	< 0.0001[a]
X_1	328.52	1	328.52	332.34	< 0.0001
X_2	27.83	1	27.83	28.15	0.0011
X_3	51.41	1	51.41	52.01	0.0002
X_4	4.97	1	4.97	5.03	0.0598
X_5	4.47	1	4.47	4.53	0.0710
X_1X_2	37.39	1	37.39	37.83	0.0005
X_1X_3	16.73	1	16.73	16.92	0.0045
X_1X_4	11.70	1	11.70	11.83	0.0108
X_2X_3	6.15	1	6.15	6.22	0.0413
X_2X_4	33.76	1	33.76	34.15	0.0006
X_2X_5	50.48	1	50.48	51.07	0.0002
X_3X_4	38.25	1	38.25	38.70	0.0004
X_3X_5	36.48	1	36.48	36.91	0.0005
Curvature	682.05	1	682.05	689.99	< 0.0001[a]
Residual	6.92	7	0.99		
Lack of fit	1.43	2	0.71	0.65	0.5608[b]
Pure error	5.49	5	1.10		
Cor total	1337.10	21			
R^2	0.9894				
R	0.9946				
Adjusted R^2	0.9698				

Prob > *F* less than 0.050 indicate model terms are significant
[a] model and curvature are significant, [b] lack of fit is not significant

In addition, the determination coefficient R^2 of the model was 0.9894 indicating that 98.94% of the variability in the observed results was demonstrated by the model, which was found to be highly significant. Furthermore, the curvature *Prob* > *F*-value of <0.0001 indicated that the curvature in the design space was greatly significant relative to the noise. The lack of fit (LOF) value was insignificant (*Prob* > *F* = 0.5608) in relation to the pure error. The significant value of curvature and insignificant LOF value indicated that the developed model was a good fit. It was also found that the main effect

of reaction time (X_1), temperature (X_2), pH (X_3) and the two level interactions of X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_2X_5, X_3X_4, and X_3X_5 were significant model terms. Other terms such as X_4 (NADPH concentration) and X_5 (enzyme concentration), two level interactions of X_1X_5, X_4X_5, and higher level (>2) interactions (such as $X_1X_2X_3$) were insignificant in improving xylitol yield as their confidence level were less than 95% (*Prob* > *F* = > 0.050 indicate model terms are not significant). Based on the magnitude of *F*-value, the ranking of the significant model terms in the FFD study is $X_1 > X_3 > X_2X_5 > X_3X_4 > X_1X_2 > X_3X_5 > X_2X_4 > X_2 > X_1X_3 > X_1X_4 > X_2X_3$. The statistical significance of the main and interaction effects of independent variables on the response were further diagnosed and compared, and graphically presented in the half-normal probability plot (Figure 6.8). It was observed that the ranking of dominating effects that are likely to identify the influential variables were essentially consistent with the output of ANOVA (Table 6.3). Thus, the reaction time, temperature and pH are most significant factors influencing xylitol production, especially the reaction time (*Prob* > *F* = < 0.0001).

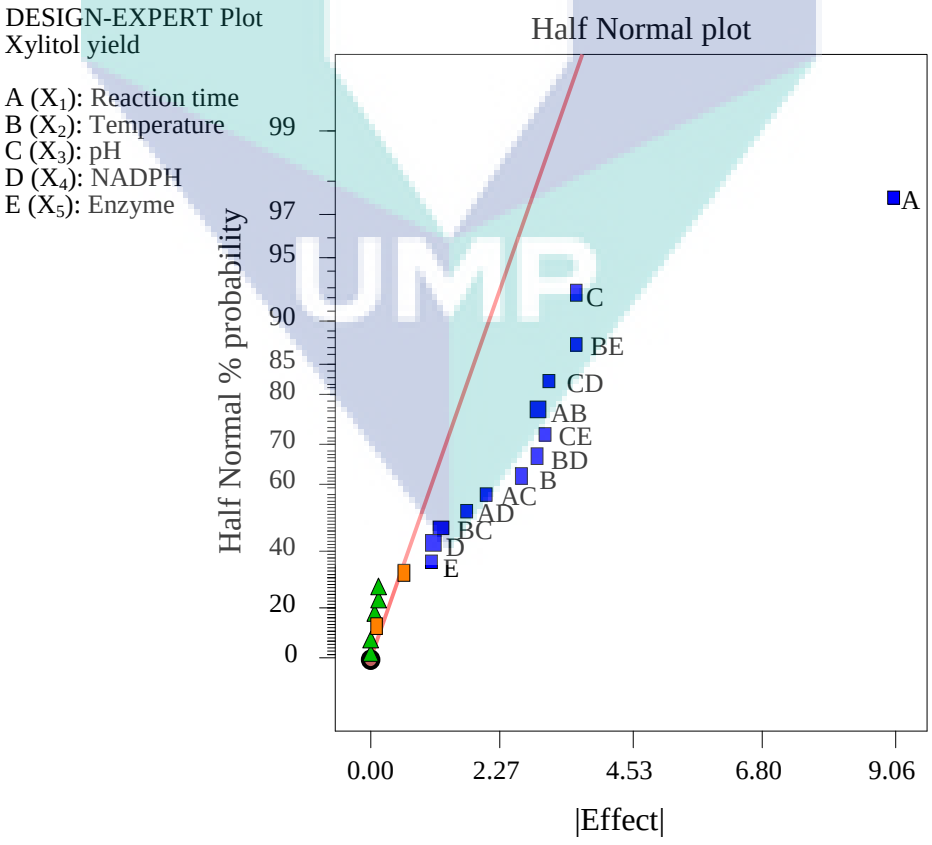


Figure 6.8: Half normal plot of effects for 2^{5-1} fractional factorial design

The first order model created from FFD analysis could be employed to screen the crucial and critical factors of experimental conditions. It is mentioned that a factor is treated to have higher significant influence on xylitol yield if its coefficient value is relatively greater than the others. Furthermore, a factor with a positive coefficient has an enhancing impact towards xylitol yield compared to a negative value, which had the opposite impact. A first order (factorial design) model (Eq. (6.1)) in terms of coded variables was obtained from the regression results, and statistically insignificant terms were excluded from the equation to make a simpler model. From Eq. (6.1) it is inferred that the main effect of reaction time has the largest coefficient (X_1; +4.53) followed by pH (X_3; +1.79), temperature (X_2; +1.32), enzyme concentration (X_5; –0.53) and NADPH concentration (X_4; –0.56). These results were closely consistent with ANOVA output (Table 6.3) where the variable reaction time (X_1) has the highest *F*-value.

$$\begin{aligned}\text{Xylitol yield}\,(\%) = {} & +60.75 + 4.53X_1 + 1.32X_2 + 1.79X_3 - 0.56X_4 - 0.53X_5 \\ & + 1.53X_1X_2 - 1.02X_1X_3 - 0.85X_1X_4 + 0.62\ X_2X_3 \\ & + 1.45X_2X_4 + 1.78X_2X_5 - 1.55X_3X_4 + 1.51\,X_3X_5\end{aligned} \qquad (6.1)$$

Interactions among Variables in Screening Design

The interactions among different variables involved in FFD were assessed by plotting the interaction and response surface curves for maximum xylitol production. The interaction and surface plots were constructed showing the interaction among two factors by holding others at their middle level for the prediction of xylitol yield (Figure 6.9). Figure 6.9(a_1, a_2) highlights how the xylitol yield was influenced by the interactive effect between reaction time (X_1) and temperature (X_2) in screening design when other three parameters pH (X_3), NADPH concentration (X_4), and enzyme concentration (X_4) were fixed at 6.0, 2.83 g/L, and 3% (v/v), respectively. It was found that xylitol yield increased while the reaction time and temperature increased to maximum level (Figure 6.9(a_1, a_2)). The figure also depicts a remarkable enhancement in xylitol yield due to the interaction among reaction time and temperature (X_1X_2) as the reaction time increases from 8 to 12 h, suggesting that this parameter has significant effect on the yield. The improvement in yield (from 56.01% for 8 h to 68.13% for a reaction time of 12 h) brought by increasing reaction time appeared to be larger at higher temperature

conditions (35 ºC). These findings implied that long reaction time will result in higher xylitol yield (71.12%) as demonstrated by run 4 (Table 6.2).

The interaction among reaction time and pH (X_1X_3) for xylitol yield at middle levels of temperature, NADPH, and enzyme concentration (30 ºC, 2.83 g/L, and 3%, respectively) is presented in Figure 6.9(b_1, b_2). The results essentially interpreted that increasing the pH at longer reaction time (12 h) results in appreciable increase in xylitol yield from 64.52% at pH 5.0 to 66.05% at pH value of 7.0 (Figure 6.9(b_1, b_2)). On the other hand, increase in pH at shorter reaction time (8 h) seemed to have minor impacts on the enhancement of xylitol yield (from 53.40% at pH 5.0 to 59.04% at pH value of 7.0). These observations could be explained in terms of the limited ionization of functional groups of XR at low pH condition (Nidetzky et al., 2003). Based on these outcomes, it can be concluded that high pH value offers the highest xylitol yield of 66.44% as achieved in run 14 (Table 6.2).

The interaction plot and its corresponding response surface plot representing the effect of interaction between temperature and pH (X_2X_3) on xylitol yield when reaction time, NADPH concentration, and enzyme concentration were set at 10 h, 2.83 g/L, and 3%, respectively, is depicted in Figure 6.9(c_1, c_2). It was observed that increasing the reaction temperature from 25 to 35 ºC, xylitol yield increased progressively from 60.61% at 25 ºC to 64.48% at 35 ºC at high pH value (pH 7.0) (Figure 6.9(c_1, c_2)). Under the same temperature, a little improvement in xylitol yield was seen at low pH value (pH 5.0) (increased from 58.26% at 25 ºC to 59.66% at 35 ºC). This outcome demonstrated that the enzyme efficiency for xylose to xylitol bioconversion is synergistically influenced by temperature and pH of the reaction. It can be highlighted that higher temperature will produce the maximum xylitol yield (68.03%) as determined by run 12 (Table 6.2).

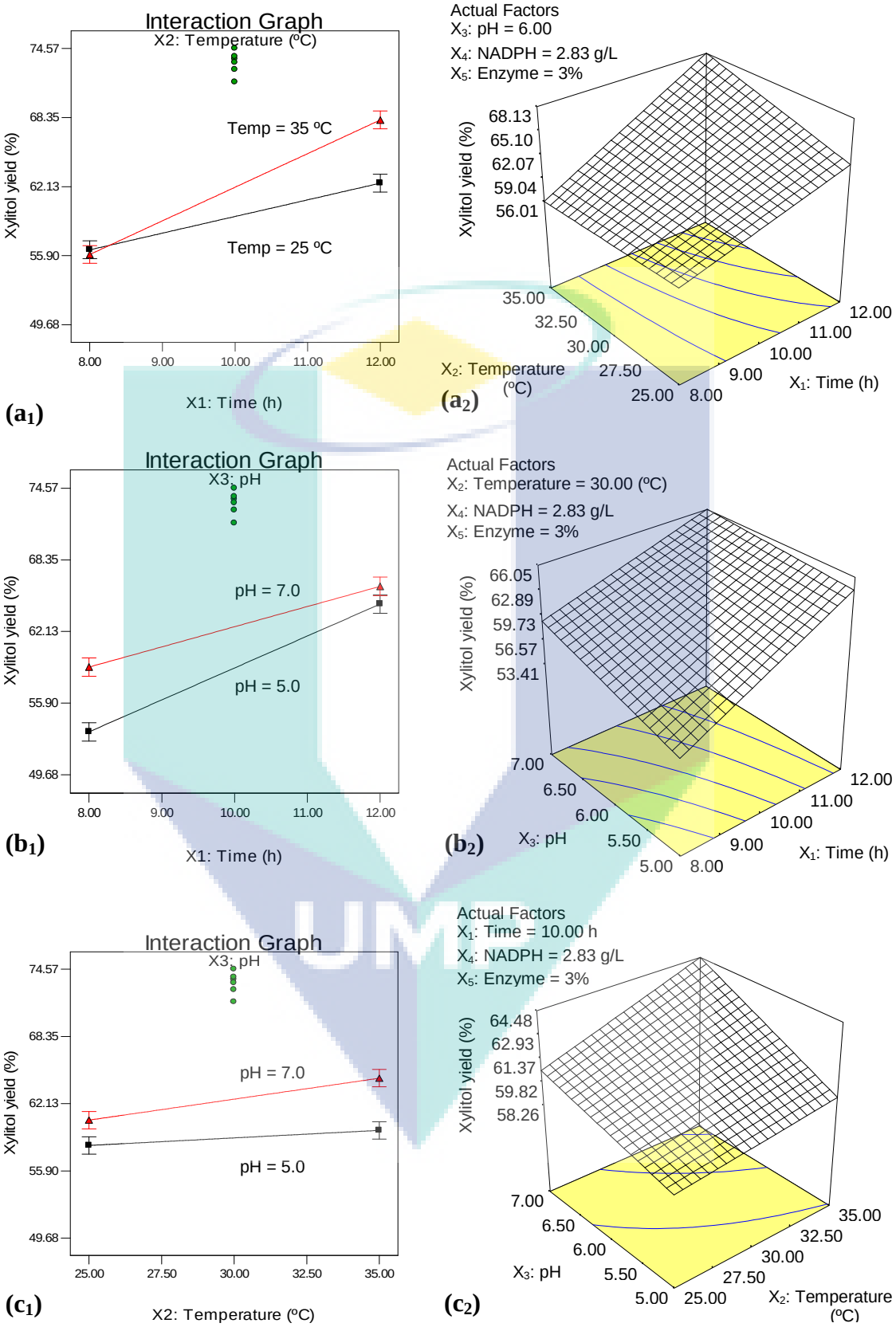


Figure 6.9: Plots of interaction effects for xylitol yield in screening process: (a_1, a_2) effect between reaction time and temperature, (b_1, b_2) effect between reaction time and pH, (c_1, c_2) effect between temperature and pH

The suitable conditions for the highest xylitol synthesis determined by FFD study were reaction time 12 h, temperature 35 ºC, pH 7.0, NADPH concentration 2.83 g/L and enzyme concentration 3% (v/v). These conditions led to a xylitol production of 13.37 g/L with a yield of 71.11%. Among the variables screened out through FFD, reaction time (X_1), temperature (X_2) and pH (X_3) were identified as the most crucial variables influencing xylitol synthesis. NADPH concentration and enzyme concentration in the production of xylitol did not yield remarkable variation at 95% confidence level (CL). Based on the $P > F$-values, it could be demonstrated that three interactive terms (X_1X_2, X_1X_3 and X_2X_3) were also crucial at 95% of CL. Hence, the three variables reaction time, temperature and pH were selected and used for further optimization by RSM. The insignificant factors such as NADPH concentration and enzyme concentration in the subsequent CCD experiments were set at their middle levels of 2.83 g/L and 3%, respectively. It is important to note here that the number of variables to be considered in the optimization design is significantly reduced from 5 to 3. Thus FFD proved to be an important statistical design which can be employed to determine the most influential factors and their interactions that act more on experiments with a reduced number of runs in a bioprocess. Therefore, the obtained first order regression model was found useful for further optimization studies involving central composite design (CCD) under RSM in order to create a second order model, which can predict the responses more accurately.

6.2.3 Optimization of Xylitol Synthesis by RSM

Enzyme-based xylitol synthesis can be enhanced by fine tuning of various parameters involved in the bioconversion reaction. Therefore, further optimization study on their effect following statistical approach is very important in order to improve the yield and productivity. RSM is a powerful mathematical and statistical tool useful for modelling and analyzing the problems related to response of interest influenced by different variables (Montgomery, 2001). As shown earlier (Table 6.2), the yields of xylitol varied markedly from 49.68–74.57% under different levels of reaction parameters. This longer variation reflected the importance and necessity of parameter optimization using RSM to attain maximum xylitol production. Till now, no study has been done on the application of RSM to optimize enzymatic production of xylitol. Once

the critical parameters (reaction time, temperature and pH) were screened by FFD, CCD under RSM was performed to develop second order model and to fine-tune the most important variables for enhanced xylitol production from MWSHH using XR. Table 6.4 presents the experimental matrix including values of responses $Y_{p/s}$ (%) and Q_P (g/L·h), where $Y_{p/s}$ (xylitol yield) was defined by [(g of xylitol produced/g of xylose consumed at the end of each run) × 100] and Q_P (productivity), defined by [xylitol concentration (g/L)/overall reaction time (h) ratio]. The outcomes of the RSM study on the bioproduction of xylitol are documented below.

Table 6.4: Experimental design and results of the CCD for xylitol synthesis

Run No.	Coded variables			Actual input variables			Responses	
	X_1	X_2	X_3	Time (h)	Temp (°C)	pH	$Y_{p/s}$ (%)	Q_p (g/L·h)
1	-2	0	0	10	35	6.5	63.52 ± 2.34	1.19 ± 0.04
2	1	1	1	13	37	7.0	77.06 ± 2.77	1.11 ± 0.04
3	0	0	0	12	35	6.5	84.73 ± 2.94	1.33 ± 0.06
4	0	2	0	12	39	6.5	76.09 ± 3.43	1.19 ± 0.05
5	1	1	-1	13	37	6.0	76.88 ± 3.44	1.11 ± 0.05
6	0	0	-2	12	35	5.5	63.13 ± 2.34	0.99 ± 0.04
7	-1	1	1	11	37	7.0	78.88 ± 3.51	1.35 ± 0.06
8	1	-1	-1	13	33	6.0	76.21 ± 3.04	1.10 ± 0.04
9	-1	-1	1	11	33	7.0	73.22 ± 2.40	1.25 ± 0.04
10	0	0	0	12	35	6.5	85.27 ± 3.31	1.34 ± 0.05
11	0	0	0	12	35	6.5	85.53 ± 3.47	1.34 ± 0.05
12	-1	1	-1	11	37	6.0	67.73 ± 3.77	1.16 ± 0.06
13	0	0	0	12	35	6.5	84.75 ± 3.75	1.33 ± 0.06
14	2	0	0	14	35	6.5	73.34 ± 3.82	0.98 ± 0.05
15	-1	-1	-1	11	33	6.0	62.91 ± 2.42	1.08 ± 0.04
16	0	0	0	12	35	6.5	84.11 ± 4.17	1.32 ± 0.07
17	1	-1	1	13	33	7.0	72.87 ± 2.31	1.05 ± 0.03
18	0	0	0	12	35	6.5	84.98 ± 4.88	1.33 ± 0.08
19	0	-2	0	12	31	6.5	69.21 ± 1.95	1.08 ± 0.03
20	0	0	2	12	35	7.5	72.16 ± 4.25	1.13 ± 0.07

$Y_{p/s}$ = xylitol yield (%), Q_p = xylitol volumetric productivity (g/L·h)

Optimization of Reaction Condition for Xylitol Synthesis

The CCD was applied to examine the mutual interactions among the most significant input variables reaction time (X_1), temperature (X_2), and pH (X_3), and to determine the exact optimum values of variables taking xylitol yield and productivity as output variables or responses. The input and output variables were fitted to the second order equation and investigated in terms of the goodness of fit of the model. The fitness of the model was verified by different criteria such as *F*-value, *P*-value, determination coefficient (R^2), and correlation coefficient (R). Table 6.5 summarizes the ANOVA results of response surface quadratic models for xylitol yield ($Y_{p/s}$) and productivity (Q_P). The *F*- and *P*-values are used as tools to check the significance of each model term coefficient and the interaction strength of parameters. The larger *F*- values corresponding to smaller *P*-values indicate more significant effect of the corresponding coefficients or model terms (Montgomery, 2001). The ANOVA for xylitol yield (Table 6.5) demonstrated that the model was quite significant, as was evident from the high *F*-value (F_{model} = 511.80) and very small probability value ($P < 0.0001$). These outcomes ensured a satisfactory adjustment of the model to the experimental data and implied that the model terms have a significant impact on the response.

Furthermore, the determination coefficient (R^2) was 0.9878, which indicates that 98.78% of the sample variation in the xylitol yield is attributed to the input variables. The value of R^2 also indicates that only 1.22% of the total variation was not explained by the model, which is accounted as residual. As stated by Montgomery (2001), closer the R^2 value to 1.0, stronger the model and better the response predictions. The value of R for xylitol yield was 0.9939, implying a satisfactory correlation among the observed and predicted results. The quadratic effect of pH (X_3^2), reaction time (X_1^2), temperature (X_2^2), the linear effect of reaction time (X_1), pH (X_3), and the interaction effect of reaction time and temperature (X_1X_3) were the primary determining factors of the response in xylitol yield ($Y_{p/s}$) as they had the largest coefficient values (Eq. (6.2)). Meanwhile, the linear effect of temperature (X_2), and the interaction effect of reaction time and temperature (X_1X_2), temperature and pH (X_2X_3) were the secondary determining factors with medium coefficients. Among them, the linear terms X_1, X_2 and X_3, and the interaction term X_2X_3 had positive coefficient implying a favorable effect on

the response. The negative coefficient indicates an adverse impact on the response. According to the magnitude of *F*-value, the order of effects of model terms on xylitol yield is $X_3^2 > X_1^2 > X_2^3 > X_1 > X_3 > X_1X_3 > X_2 > X_1X_2 > X_2X_3$.

Based on the outcomes of ANOVA (Table 6.5), the *F*-value of the model representing xylitol productivity was 496.60 with a very low probability value of <0.0001, indicated that the model was highly significant. The value of R^2 was 0.9867, implying that only 1.33% of the variations are not interpreted by the model. The *R* value was estimated to be 0.9933, implying a better correlation between the actual and predicted values of xylitol productivity. Notably, the insignificant lack of fit ($P > 0.050$) was observed for both regression equations and thus ensuring a satisfactory fitness of models to the experimental data (Table 6.5). The adjusted determination coefficients (adj R^2) for xylitol yield and productivity were also found very high (0.9859 and 0.9854, respectively) indicating that the created models were greatly significant and suitable for use in this experiment. From the regression coefficient values (Eq. (6.3)), it can be concluded that all the linear terms X_1, X_2, X_3, their quadratic terms and interaction terms except X_2X_3 had significant effect on xylitol productivity. The order of significant effects (based on *F*-value) of terms on xylitol productivity is found to be the same as ranked for the xylitol yield. The second order equations in terms of coded factors for xylitol yield (Eq. (6.2)) and productivity (Eq. (6.3)) were derived from regression analysis of data.

$$Y_{p/s} = +84.86 + 2.49X_1 + 1.82X_2 + 2.27X_3 - 4.13X_1^2 - 3.08X_2^2 - 4.33X_3^2 - 0.70X_1X_2 - 3.08X_1X_3 + 0.55X_2X_3 \quad (6.2)$$

$$Q_p = +1.33 - 0.056X_1 + 0.029X_2 + 0.037X_3 - 0.062X_1^2 - 0.049X_2^2 - 0.068X_3^2 - 0.014X_1X_2 - 0.051X_1X_3 + 0.009X_2X_3 \quad (6.3)$$

where $Y_{p/s}$ and Q_p are the predicted responses of xylitol yield and productivity, respectively; X_1, X_2, and X_3 are the variables time, temperature, and pH, respectively. The empirical equations constructed are mathematical correlation models that can be used to navigate the design space to predict and optimize the targeted responses.

Table 6.5: ANOVA for the quadratic model adjusted to xylitol yield ($Y_{p/s}$) and volumetric productivity (Q_P)

Source	Sum of squares		Degree of freedom		Mean square		*F*-value		*Prob > F*	
	$Y_{p/s}$	Q_P	$Y_{p/s}$	Q_P	$Y_{p/s}$	Q_P	$Y_{p/s}$	Q_P	$Y_{p/s}$	Q_P
Model	1127.56	0.30	9	9	125.28	0.034	511.80	496.60	< 0.0001[a]	< 0.0001[a]
X_1	99.60	0.050	1	1	99.60	0.050	406.88	731.58	< 0.0001	< 0.0001
X_2	52.93	0.014	1	1	52.93	0.014	216.21	204.02	< 0.0001	< 0.0001
X_3	82.63	0.022	1	1	82.63	0.022	337.54	321.50	< 0.0001	< 0.0001
X_1^2	429.50	0.096	1	1	429.50	0.096	1754.54	1419.87	< 0.0001	< 0.0001
X_2^2	238.22	0.061	1	1	238.22	0.061	973.14	903.71	< 0.0001	< 0.0001
X_3^2	471.25	0.12	1	1	471.25	0.12	1925.11	1721.49	< 0.0001	< 0.0001
X_1X_2	3.95	1.513E-003	1	1	3.95	1.513E-003	16.13	22.35	0.0025	0.0008
X_1X_3	75.77	0.021	1	1	75.77	0.021	309.52	310.51	< 0.0001	< 0.0001
X_2X_3	2.38	6.125E-004	1	1	2.38	6.125E-004	9.71	9.05	0.0110	0.0132
Residual	2.45	6.767E-004	10	10	0.24	6.767E-05				
Lack of fit	1.23	3.934E-004	5	5	0.25	7.867E-05	1.01	1.39	0.4942[b]	0.3638[b]
Pure error	1.22	2.833E-004	5	5	0.24	5.667E-05				
Cor total	1130.01		19	19						
R^2	0.9878	0.9867								
R	0.9939	0.9933								
Adj R^2	0.9859	0.9854								

Prob > F less than 0.050 indicate model terms are significant
[a] model is significant, [b] lack of fit is not significant

Interaction of Variables during Optimization by RSM

The estimation of xylitol yield and volumetric productivity over the most important input variables reaction time, temperature, and pH in the forms of interaction and response surface plots are presented in Figures 6.10–6.12 and 6.13–6.15, respectively. The interaction plots (two dimensional) and their respective response surface plots (three dimensional) are the graphical representations of the regression model equation developed to locate the optimum values of the input variables within the selected ranges for maximizing response of a production process (Montgomery, 2001 and Tanyildizi et al., 2005). The response surface with an elliptical or saddle nature of the contour diagram indicates the significant interaction among the corresponding variables, whereas with circular contour diagram indicates the negligible interaction (Liu et al., 2010 and Muralidhar et al., 2001). The variables tested in CCD had most significant influence on xylitol synthesis as evidenced by their *P*-values (*P*-values less than 0.050 indicate that model terms are significant) (Table 6.5). The interaction of reaction time and temperature (X_1X_2), reaction time and pH (X_1X_3), and temperature and pH (X_2X_3) are common effects for xylitol yield and productivity, which were found to remarkably affect the results of both responses. Based on coefficient- and *F*-values, the contribution order of interaction effects for xylitol yield and productivity is identical and expressed as $X_1X_3 > X_1X_2 > X_2X_3$. The mutual interactions of crucial variables during optimization of xylitol synthesis by RSM are detailed below:

Figure 6.10 shows the interaction effect of reaction time and temperature on xylitol yield when pH was maintained at 6.5 as the centre point. The analyses of interaction and response surface plots showed that the predicted xylitol yield slowly increased with increase in reaction time and reached a maximum value (from 77.68 % at 11 h to 81.26% at 13 h) while the temperature was kept at 37 ºC (Figure 6.10(a, b)). A similar pattern of curves was observed at low temperature (33 ºC) where the yield enhanced rapidly from 72.63% at 11 h to 79.03% at 13 h. The longer reaction time gave maximum xylitol yield probably because of the adequate time required for the enzyme to contact as well as react with substrate (xylose). As reported by Mussatto et al. (2008), the enzymatic reaction requires certain period for direct physical contact and binding between enzyme and substrate in order to product formation. These results highlighted

that longer reaction time will be favorable to attain the highest xylitol yield (77.06%) that is resulted in the experimental run 2 (Table 6.4). The relatively long (14 h in run 14) or short (10 h in run 1) reaction time led to reduction in xylitol yield to 73.34 and 63.52%, respectively.

The interaction relationship between reaction time and pH for xylitol yield at a temperature of 35 ºC is presented in Figure 6.11. A notable and constant improvement in xylitol yield was observed (Figure 6.11(a, b)) for the increase of reaction time (from 68.55% at 11 h to 79.70% at 13 h) while holding pH at low level (6.0). At high pH condition (pH 7.0), a complementary result was also observed where the yield of xylitol decreased slightly from 79.25% at 11 h to 78.09% at 13 h of reaction. These results probably indicate that a near-neutral pH facilitates the ionization of XR functional groups and consequently enhances the production of xylitol. As reported by Mehrnoush et al. (2011), an enzyme generally exhibited the highest activity when the pH-dependent ionizable groups of the enzyme are in appropriate ionic forms, and a well organized active site allows unrestricted access to substrates. These findings led to the conclusion that the low initial pH is suitable for the enzymatic conversion of xylose to xylitol that is evidenced by run 8 with a maximum yield of 76.21% (Table 6.4). However, the extremely high (pH 7.5 in run 20) or low (pH 5.5 in run 6) pH decreased xylitol yield to 72.16 and 63.13%, respectively.

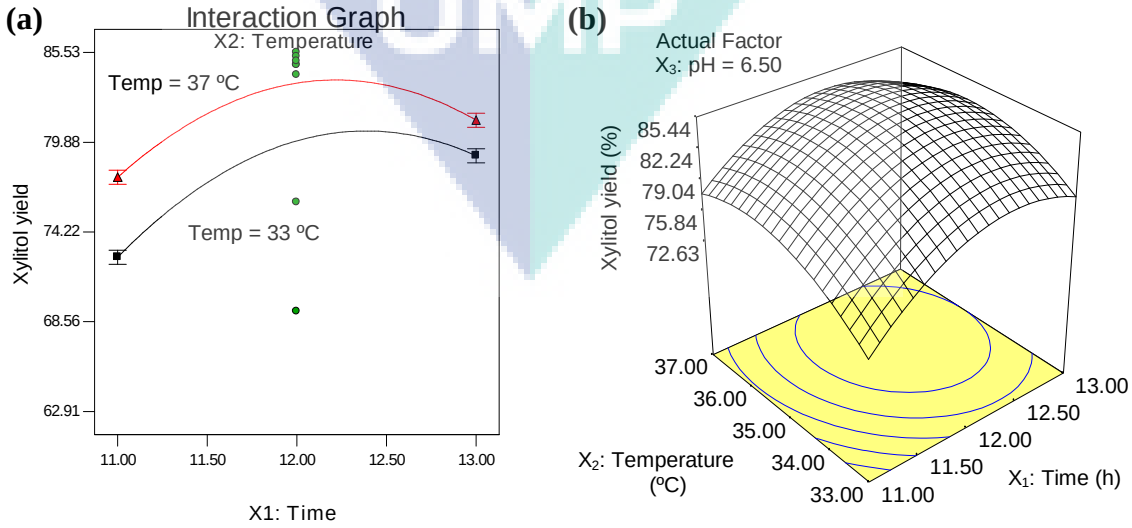


Figure 6.10: Interaction (a) and response surface plot (b) showing the effect of reaction time and temperature on xylitol yield

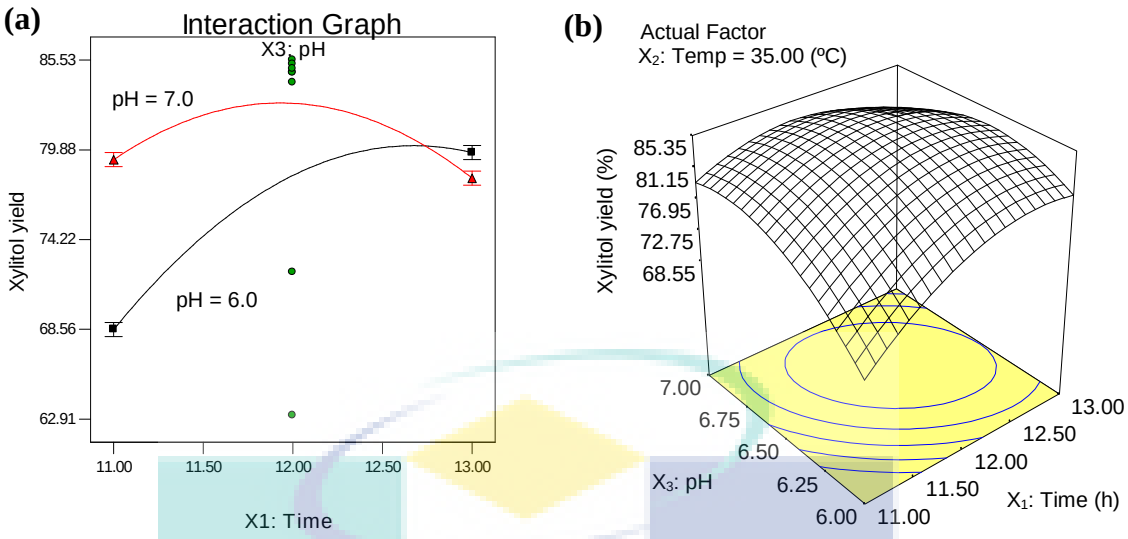


Figure 6.11: Interaction (a) and response surface plot (b) showing the effect of reaction time and pH on xylitol yield

Figure 6.12 is the interaction and response surface plot for the variation in xylitol yield as a function of temperature and pH by setting the reaction time at 12 h as the medium level. When the initial pH was fixed at a high value (pH 7.0), the predicted xylitol yield gradually improved with increasing temperature from 77.36% at 33 ºC to 82.09% at 37 ºC. Again, at a low pH (pH 6.0), the trend of xylitol yield was similar in which the yield enhanced slightly from 73.91 at 33 ºC to 76.45% at 37 ºC (Figure 6.12(a, b)). The maximum xylitol yield was obtained at high temperature possibly due to the availability of required activation energy for XR. According to Nidetzky et al. (1996) and Yokoyama et al. (1995), the reaction temperatures ranging from 25–35 ºC are optimum for the enzyme-based bioproduction of xylitol. The results obtained suggested that high temperature positively affected xylitol synthesis and provided higher yield (78.88%) as determined by run 7 (Table 6.4). At a relatively high temperature (39 ºC), xylitol yield slightly decreased to 76.09% (in run 4) and at a relatively low temperature (at 31 ºC in run 19) it markedly declined to 64.14%.

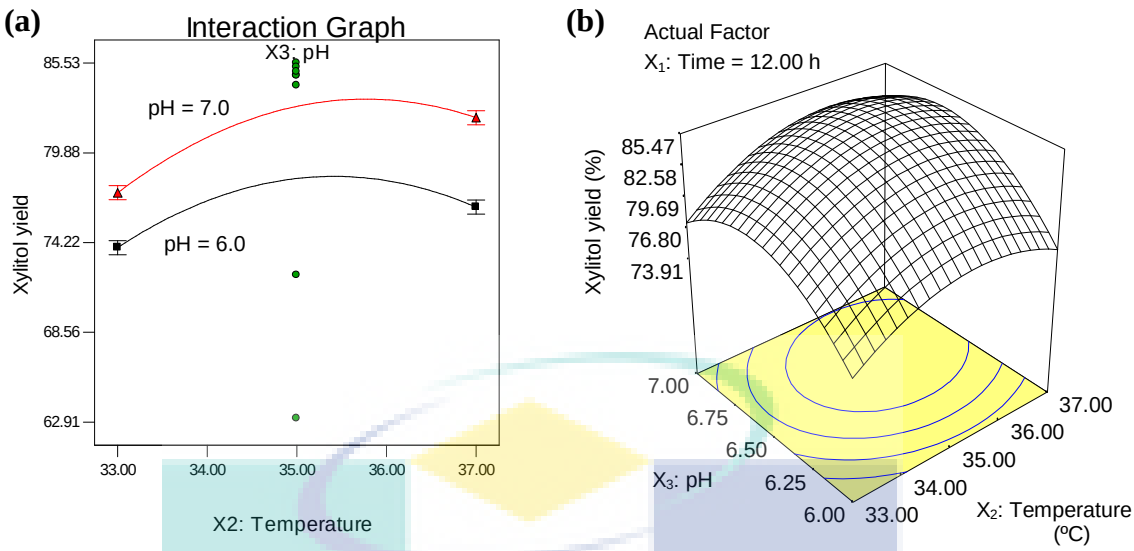


Figure 6.12: Interaction (a) and response surface plot (b) showing the effect of temperature and pH on xylitol yield

The interaction effect of reaction time and temperature on xylitol productivity at pH 6.5 is shown in Figure 6.13. It was found that the predicted xylitol productivity improved with the increase of reaction time to certain extent and then sharply reduced with further increase of time. The elliptical diagram presents that the productivity decreased from 1.32 g/L·h at 11 h to 1.18 g/L·h at 13 h while setting temperature at 37 ºC. At low temperature (33 ºC) condition, a similar phenomenon was also found in which the maximum and minimum productivities of 1.23 and 1.15 g/L·h were predicted at 11 and 13 h, respectively (Figure 6.13(a, b)). By analyzing these results, it can be concluded that short reaction time will offer higher xylitol productivity (1.35 g/L·h) as obtained by the experimental run 7 (Table 6.4). By setting reaction time at 14 h (in run 14), xylitol productivity dramatically decreased to 0.98 g/L·h. However, at a relatively short reaction time (10 h in run 1), the productivity slightly reduced to 1.19 g/L·h.

Figure 6.14 depicts the influence of reaction time and pH on xylitol productivity when temperature was held constant at 35 ºC. At high pH (pH 7.0), increase in reaction time led to a rapid decrease in xylitol productivity from 1.34 g/L·h at 11 h to 1.13 g/L·h at 13 h. At a low pH (pH 6.0) condition, xylitol production rate increased with increase in reaction time up to its middle level (12 h), and then decreased back to the initial value (1.16 g/L·h at 11 h) (Figure 6.14(a, b)). These results demonstrated that high pH

condition facilitated the XR-catalyzed conversion of xylose to xylitol and contributed the highest productivity (1.25 g/L·h) as attained by run 9 (Table 6.4). By using the highest pH value (pH 7.5 in run 20), the productivity reduced to 1.13 g/L·h whereas at the lowest pH (at pH 5.5 in run 6), it sharply reduced to 0.99 g/L·h.

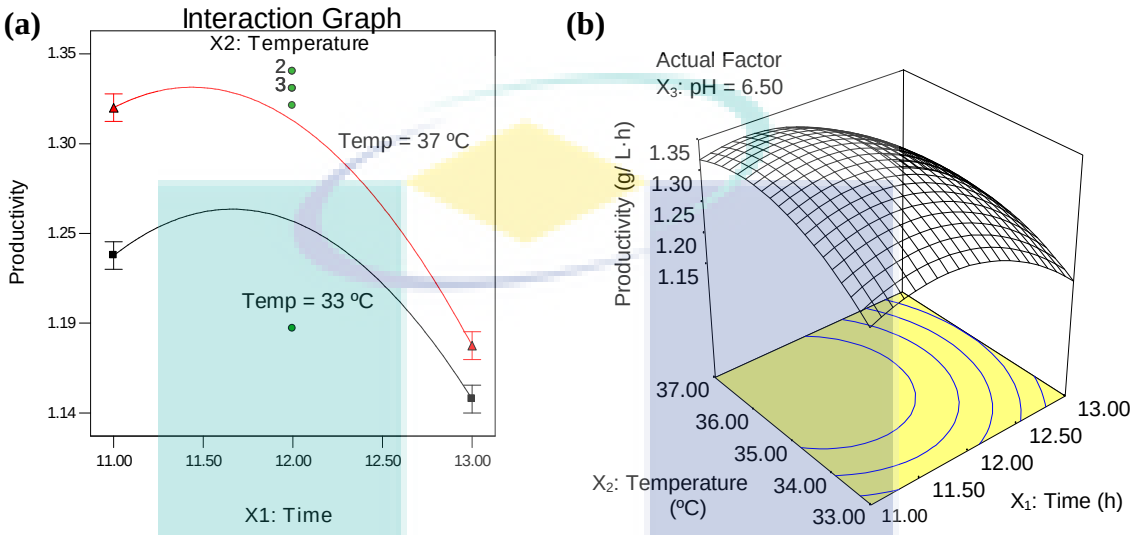


Figure 6.13: Interaction (a) and response surface plot (b) showing the effect of reaction time and temperature on xylitol productivity

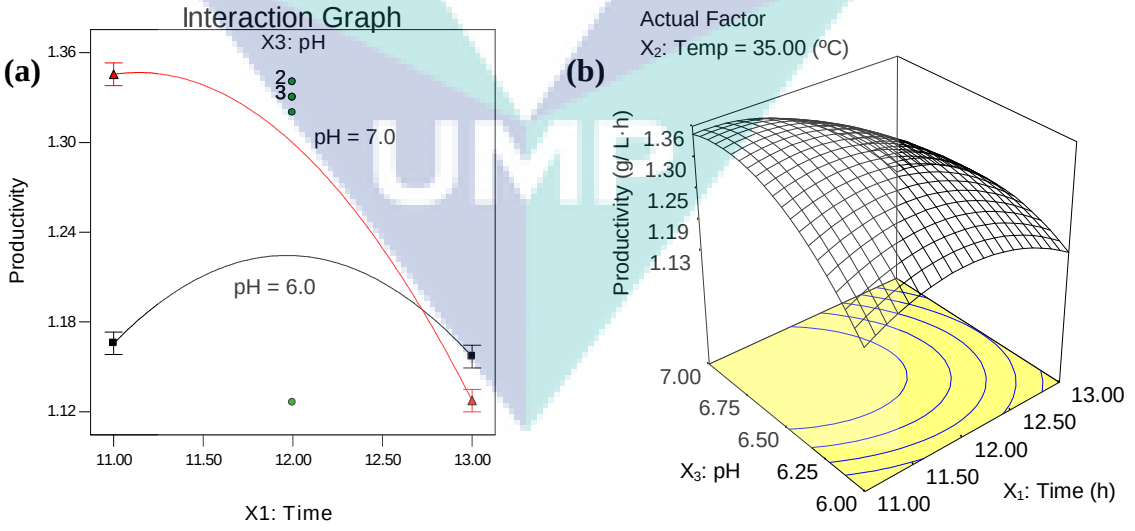


Figure 6.14: Interaction (a) and response surface plot (b) showing the effect of reaction time and pH on xylitol productivity

Figure 6.15 presents the interactive effect of temperature and pH on xylitol productivity while keeping reaction time at 12 h as the centre point. As shown in Figure 6.15(a, b), the predicted xylitol productivity increased with the increase of temperature from 1.21 g/L·h at 33 ºC to 1.32 g/L·h at 37 ºC when pH was maintained at 7.0. At low pH (pH 6.0), a reciprocal phenomenon were observed where a little improvement in productivity occurred (from 1.16 g/L·h at 33 ºC to 1.19 g/L·h at 37 ºC). These outcomes indicated that high temperature resulted in higher productivity (1.35 g/L·h) as interpreted by run 7 (Table 6.4). However, xylitol productivity decreased to 1.19 and 1.08 g/L·h at relatively high (at 39 ºC in run 4) or low (at 31 ºC in run 19) temperatures, respectively.

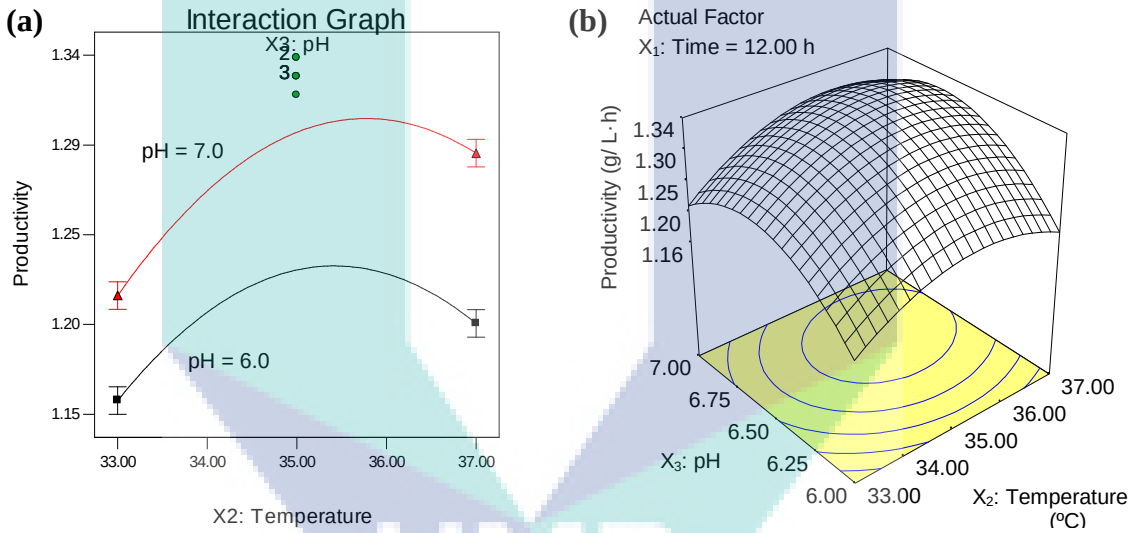


Figure 6.15: Interaction (a) and response surface plot (b) showing the effect of temperature and pH on xylitol productivity

Validation of Model for Xylitol Synthesis

Model validation is an important step of optimization procedures and is used to verify the acceptability, adequacy and accuracy of the constructed model. Numerical optimization was conducted based on the regression models for measuring xylitol yield ($Y_{p/s}$) and productivity (Q_p) using Design Expert software. Five proposed optimum conditions were chosen considering those levels of variables that led to maximum responses ($Y_{p/s}$ and Q_p) with higher desirability factor (0.9589). Validation experiments were performed at suggested optimum conditions and repeated three times to justify these conditions. The proposed optimum conditions with actual and predicted results are summarized in Table 6.6. The analyses of residuals and percentage errors were done by comparing the observed value and corresponding predicted value of both responses from validation trails to check the accuracy of models. The residuals and percentage errors were computed based on Eqs. (5.9) and (5.10) (as mentioned in subsection 5.2.3 of chapter 5). The combination of parameter settings selected for a maximum xylitol production were reaction time 12.25 h, temperature 35 ºC, and pH 6.5. Under these conditions, 86.57% of xylitol yield was obtained by experiments with a production rate of 1.33 g/L·h, which are recorded in boldface in Table 6.6. The percentage errors for both xylitol yield and productivity are ranging from 1.24–4.31% (Table 6.6), which implied that the created models were accurate sufficiently because the errors were well within acceptable value (5%). These findings also implied that the developed models were to be accurate and reliable for predicting the yield and productivity of xylitol within the 95% confidence interval (CI).

Table 6.6: Results of model validation for xylitol yield and productivity

Run	Variables			Xylitol yield ($Y_{p/s}$; %)				Productivity (Q_p; g/L·h)			
	Time (h) (X_1)	Temp (°C) (X_2)	pH (X_3)	Actual	Predicted	Residual	Error (%)	Actual	Predicted	Residual	Error (%)
1	**12.25**	**35**	**6.5**	**86.57**	**85.36**	**+1.21**	**1.40**	**1.33**	**1.31**	**+0.02**	**1.40**
2	12.25	35	6.6	86.41	85.34	+1.07	1.24	1.33	1.31	+0.02	1.24
3	12.25	35	6.4	83.18	85.36	-2.18	2.62	1.28	1.31	-0.03	2.62
4	12.25	35	6.7	81.81	85.34	-3.53	4.31	1.26	1.31	-0.05	4.31
5	12.25	35	6.3	86.54	85.35	+1.19	1.38	1.33	1.31	+0.02	1.38

Model Confirmation Testing

Confirmation testing is the final step of response surface study and is important to prove the developed model directly. A confirmation run consists of adopting the suggested levels of the critical variables and the most favorable settings of all remaining variables studied in the experiment. Based on the validation results, the optimum conditions for xylitol production were chosen with the use of a reaction time of 12.25 h, temperature at 35 ^{o}C and an initial pH of 6.5. The experimental and predicted results of confirmation tests are reported in Table 6.7. The predicted values of xylitol yield and productivity under the aforementioned optimum conditions were 85.36% and 1.31 g/L·h, respectively, at 95% of the CI. These values of responses were confirmed by batch enzymatic reaction in triplicate sets of conformation experiments. The average values of xylitol yield and volumetric productivity obtained were 86.57% and 1.33 g/L·h, respectively, which were in good agreement with the values predicted by the model in a 95% CI. These results proved that the model fitted to the experimental data. Thus, the selected optimum conditions were the most suitable in practice for enzymatic synthesis of xylitol from hemicellulosic hydrolysate.

Table 6.7: Results of confirmation run for xylitol synthesis

Optimum conditions		**Xylitol yield ($Y_{p/s}$; %)**			**Productivity (Q_p; g/L·h)**		
		Actual	**Predicted**	**Error (%)**	**Actual**	**Predicted**	**Error (%)**
Reaction time (h)	12.25						
Temperature (ºC)	35	86.57	85.36	1.40	1.33	1.31	1.40
pH	6.5						

The true optimum conditions for the synthesis of xylitol were found as reaction time for 12.25 h, temperature at 35 ºC and pH 6.5 (0.1 M phosphate buffer). These conditions resulted in a maximum xylitol production of 16.28 g/L, with a yield and productivity of 86.57% and 1.33 g/L·h, respectively. It is noted that this is the first report on the application of CCD to improve xylitol production from hemicellulosic hydrolysate using XR. From the sequential optimization studies, it was found that the xylitol yield of 86.57% with optimum conditions obtained by the CCD, which was 1.55-fold and 1.22-fold higher than the yield achieved by the OFAT (56.01%) and the FFD (71.11%), respectively. These findings indicated the significance and applicability of the sequential optimization strategies for the improvement of xylitol production. It is also noted that an 86.57% of xylose to xylitol conversion achieved under optimized conditions. This outcome interpreted that the operating conditions employed led solely xylose to xylitol transformation instead of being converted to arabinitol by XR. This fact also interpreted that MWSHH did not cause undesired reaction with the enzyme, although the enzymatic reaction was affected by the concentration of hydrolysate due to the increased amounts of toxic compounds. Nidetzky et al. (1996) studied on enzyme-based production of xylitol from commercial xylose and reported a yield of 96% and productivity of 3.33 g/L·h. Based on the literature information, the chemical process of xylitol synthesis has a yield of about 50–60% of the initial xylose (Nigam and Singh, 1995 and Parajó et al., 1998a), and for the microbiological method, 65–85% of yield has been reported (Branco et al., 2009 and Nigam and Singh, 1995). This study innovatively developed a reaction medium utilizing crude MWSHH to produce xylitol at a considerable level. However, one should keep in mind that the xylitol yield and productivity recorded in the current study might still be markedly enhanced by further study on enzyme inhibitors and application of immobilization process.

Xylitol and Byproducts in the Reaction Mixture

Lignocellulosic hydrolysates are always a cheap source of sugars but difficult to be utilized due to their complexity on inhibitory components, which can hamper a bioprocess (Branco et al., 2009 and Martínez et al., 2003). To assess the effect and potential of MWSHH as xylose source on xylitol synthesis, fixed volume of MWSHH were applied in the reaction media. A time course of changes in product and reactant

contents during enzymatic conversion under optimized conditions is shown in Figure 6.16. This time course was typical for bioconversion of xylose to xylitol as determined by RSM (as described above). During enzymatic reaction, a concomitant reduction in the concentration of xylose was found in the course of xylitol formation (Figure 6.16(a)). The highest xylitol production was achieved at a xylose concentration of 18.8 g/L. This outcome interpreted that no substrate inhibition occurred for a xylose concentration of 18.8 g/L, and the conversion of xylose to xylitol progressed at maximum speed. At concentrations above 18.8 g/L, the production of xylitol is less favored (as investigated above under OFAT study) probably because of the presence of various toxic compounds at high levels. The contents of glucose and arabinose in the reaction mixture remained almost the same throughout the reaction period. Acetic acid concentration slightly decreased with increasing reaction time probably due to its partial evaporation during the reaction. Figure 6.16(b) shows the variation in the concentrations of furfural, HMF and LDPs during reaction. It was observed that the concentration of furfural, HMF and LDPs remained almost unchanged in the reaction mixture.

A control experiment was performed using pure xylose in the optimum conditions and the process responses were compared to reaction with MWSHH. According to Figure 6.16 and Table 6.8, xylose consumption and xylitol production behaviors were somewhat different for the control and MWSHH experiments. From the Table, it was found that there was a reduction of 8.37% in $Y_{p/s}$ and 8.27% in Q_p for the experiments using MWSHH, compared to the control reaction with commercial xylose. The reduction in $Y_{p/s}$ and Q_p might be attributed to the synergistic inhibitory effect of different toxic compounds in the MWSHH. These results revealed that the MWSHH contained xylose, glucose, arabinose, acetic acid, furfural, HMF, and LDPs (in the concentrations of 18.8, 4.64, 2.55, 4.14, 0.55, 0.08, and 1.55 g/L, respectively) did not hinder the bioconversion courses. This phenomenon is noticeably advantageous for the scale-up of this bioprocess as the utilization of MWSHH presents a diminution of the bioprocess costs.

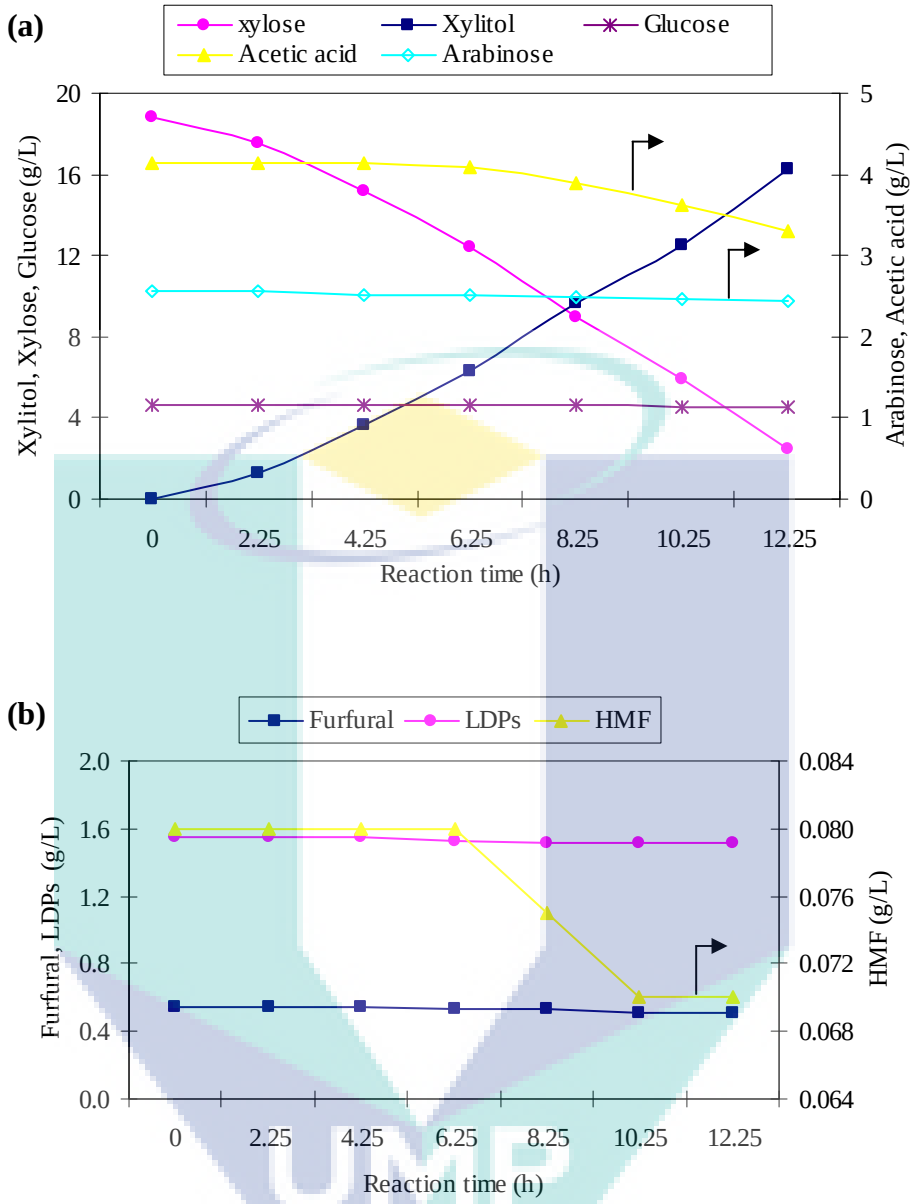


Figure 6.16: The contents of (a) xylitol, xylose, glucose, arabinose and acetic acid, and (b) furfural, HMF and LDPs in the reaction mixture under optimized conditions

Table 6.8: Xylitol yield and productivity obtained from pure xylose and from MWSHH under optimized conditions

Reaction medium	Time (h)	Xylitol conc. (g/L)	Remaining xylose (%)	Yield ($Y_{p/s}$, %)	Productivity (Q_p, g/L·h)
Pure xylose (18.8 g/L)	12.25	17.76	5.53	94.47	1.45
MWSHH (18.8 g/L)	12.25	16.28	13.40	86.57	1.33

Almeida et al. (2007) and Sarrouh et al. (2009) reported that biomass hydrolysates are consisted of various compounds formed during hydrolysis that are inhibitory to microbial metabolism and growth, and for nearly all of these compounds the individual inhibition mode and synergistic effects have not yet been completely explained. It was also reported that there are a variety of unknown components come from biomass hydrolysis that can delay a bioprocess (Almeida et al., 2007). Thus, among numerous causes, the inhibition of enzyme activity by toxic substances in the hydrolysate might be one. The activity of XR was determined at the starting and after 12.25 h of reaction, and was observed that it retained almost the same activity. In order to improve the xylitol yield and production rate, future studies need to be performed to know the reason and pattern of MWSHH inhibition on the enzymatic reaction.

6.3 Conclusion

MWSHH can be used as a promising and alternative source of xylose for the *in vitro* production of xylitol by enzyme technology. The first step of the xylitol synthesis optimization is concerned with the design of parameters following OFAT method. Xylitol production was performed in batch system from MWSHH using XR at selected operating conditions to achieve high amount of xylitol in the resulting reaction mixture. For enhanced xylitol production, the possible optimal values of variables were determined as reaction time 10 h, temperature 30 ºC, pH 7.0, xylose concentration 18.8 g/L, NADPH concentration 3.66 g/L, enzyme concentration 3% (0.33 U/mL), and agitation rate 100 rpm. These conditions led to a xylitol yield of 56.01% (w/w). It was noticed that xylitol synthesis was influenced by five variables namely reaction time, temperature, pH, NADPH concentration, and enzyme concentration. Thus, the impacts of these variables were further examined with FFD to identify the most important variables influencing xylitol synthesis. Indeed, this is the first study trying to utilize MWSHH as xylose source for enzymatic conversion to xylitol, and thus it will serve as a benchmark for further research on enzyme-based xylitol production from LCMs.

A first order model 2^{5-1} FFD was applied as a screening approach to select the critical variables considering their mutual interaction towards xylitol yield. The FFD was found to be an effective strategy to pick up the key process variables for maximum

xylitol production from MWSHH via enzymatic conversion. Using FFD, reaction time, temperature, and pH were identified as the most important factors influencing xylitol synthesis with the favorable values of 12 h, 35 ºC, and 7.0, respectively. The yield of xylitol obtained under these conditions was 71.11%. Finally, a 2^3 CCD was adopted to fine-tune the most important variables and to create statistical models for attaining optimum modes with respect to xylitol yield and productivity. The analyses of CCD and response surface were useful to determine the optimum values of critical variables that strongly influenced xylitol production. The final combinations of variables after optimization step were as follows: reaction time for 12.25 h, temperature at 35 ºC, and pH 6.5, which were justified through model confirmation testing. These optimum conditions produced theoretically a xylitol yield of 85.36% and productivity of 1.31 g/L·h and practically 86.57% of yield and 1.33 g/L·h of productivity. Hence, the obtained model was adequate to predict the optimum xylitol production from MWSHH. A 56.01% xylitol yield was obtained during parameter design by the OFAT method, which increased to 71.11% with the FFD and finally to 86.75% using CCD. This implied that optimization with sequential strategies resulted in 1.55-fold improvement in overall xylitol production. It is important to note that the use of hemicellulosic hydrolysate as a source of xylose will discourage the utilization of high priced commercial xylose and also yield a high value product from low-cost hydrolysate of MWS.

CHAPTER 7

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 INTRODUCTION

The current research was carried out to produce xylitol via enzymatic reduction of xylose from the crude MWSHH using locally produced XR as a biocatalyst. This chapter presents a brief overview of the results and discussion to summarize the research work and to furnish an overall conclusion of all the previous chapters of the thesis. The findings of this study would provide a precious enlightenment for further study on the enzymatic synthesis of xylitol as an alternative approach. Thus, some recommendations for future work are also suggested in this chapter.

7.2 GENERAL CONCLUSIONS

The potential of xylitol as a food, pharmaceutical, and cosmeceutical ingredient is well recognized. The chemical process for the manufacturing of xylitol was developed in the 1970s in Finland. Since then, the microbiological bioproduction of xylitol has been examined extensively as an alternative to the conventional chemical approach. In some of the continuous fermentation processes using wild-type xylose-fermenting yeast, a substantial improvement in xylitol productivity was achieved with moderate product yield only at low xylose concentration and high residence time. Enhancement in productivity and yield is important factor for industrial xylitol production. The fermentation method does not yet have the advantages of the chemical process due to the low initial xylose concentration used as well as to the downstream

processing problem because of the presence of the microbial media ingredients. The production of xylitol from xylose employing XR from yeast is an attractive alternative to chemical and microbial processes. The XR enzyme is not available commercially despite its potential applications in the synthesis of xylitol and ethanol. The use of XR may offer an economic interest over the chemical and microbial conversion of xylose to xylitol. One significant advantage of *in vitro* enzyme-based xylitol production is that it can afford an easy recovery of xylitol. Xylitol production by a chemical process is expensive because of the difficult separation and purification steps. On the other hand, the fermentation process on an industrial-scale is not feasible due to decreased productivity and/or yield. Therefore, it is necessary and important to explore alternative methods for the effective production of xylitol using XR. The enzymatic approach to xylitol synthesis from xylose present in the MWS biomass may provide an alternative for the chemical process. Based on the results achieved from the present study, the general conclusions can be drawn as follows:

Basically, this study was performed to utilize the sawmill waste, MWS for the bioproduction of xylitol using isolated XR. The first phase of the experiment was to characterize the MWS biomass in order to study the feasibility of using MWS as a source of xylose to be used to support the growth of yeast cells and to produce xylitol. MWS biomass was found to contain cellulose (41.06%), hemicellulose (30.64%) and lignin (22.23%) as the major biopolymers. The presence of a high amount of xylan (29.22%) in MWS renders this biomass adequate for xylose production.

The second experimental phase was tailored to evaluate several parameters that were thought to influence xylose recovery from MWS by hydrolysis. MWS hydrolysis was conducted with sulfuric acid under selected experimental conditions to ensure high xylose recovery and to minimize coformation of byproducts in the resulting hemicellulosic hydrolysate. To recover maximum xylose, optimization of MWS hydrolysis was carried out using statistical experimental design including OFAT method. Following the OFAT study, three parameters namely residence time, temperature, and acid concentration were identified as influential for xylose recovery. The highest recovery of xylose obtained at 125 ºC for 60 min using 4% H_2SO_4 and a LSR of 8 g/g, with a xylose yield of 86.3%. A kinetic study on acid hydrolysis of MWS

was executed to find kinetic parameters of mathematical models for predicting the concentration of products in the hydrolysate and for optimizing the process. It was found that optimum H_2SO_4 concentration and residence time obtained at 130 ºC were 6% (w/w) and 20 min, respectively, with the concentrations of xylose, glucose, furfural, and acetic acid, 18.5, 4.2, 0.4, and 4.1 g/L, respectively. The kinetic models obtained proved to be useful for further technical and economic studies. Based on the findings of OFAT study, the effect of three important factors (residence time, temperature, and acid concentration) was further analyzed by CCD under RSM to determine the true optimum conditions with respect to xylose yield and selectivity. The influential variables were optimized by CCD, to be residence time 80 min, temperature 124 ºC, and acid concentration 3.26%. In these conditions, xylose yield and selectivity were attained at 90.6% and 4.05 g/g, respectively. The predicted xylose yield and selectivity of 91.83% and 4.18 g/g, respectively, were very close to the observed values, indicating the models to be reasonably accurate. Therefore, the quadratic models developed could reliably be used for predicting the optimum hydrolysis conditions with respect to maximum xylose recovery from wood sawdust. The selected optimum conditions resulted in a hydrolysate containing 18.8 g/L xylose, 4.64 g/L glucose, 2.55 g/L arabinose, 4.14 g/L acetic acid, 0.55 g/L furfural, 0.08 g/L HMF, and 1.55 g/L LDPs. The hydrolysate contained a small amount of other compounds as glucose, arabinose, acetic acid, furfural, HMF, and LDPs, implying an efficient hydrolysis of MWS. Moreover, about 92% of the hemicellulose was hydrolyzed under optimized conditions. Hence, this condition was chosen, for subsequent generation of xylose-rich hemicellulosic hydrolysate to produce xylitol. The proper utilization of MWS will not only solve the disposal problem but also yield a high value product from wood industry waste.

Upon optimization of xylose recovery, this study was further extended to investigate the growth profile of *C. tropicalis* cultivated on the hydrolysate-based medium. The characterization of XR carried out in the third phase revealed that the activity of the enzyme was 11.16 U/mL. The kinetic parameters of XR for xylose reduction were calculated according to the Lineweaver-Burk method. The values of K_m for xylose and NADPH were 81.78 mM and 7.29 µM with a corresponding V_{max} for xylose and NADPH 178.57 and 12.5 µM/min, respectively. The lower K_m value for xylose indicated that XR has a higher affinity for its substrate xylose. The inhibitory

effects of six byproducts (glucose, arabinose, acetic acid, furfural, HMF, and LDPs) on XR activity were evaluated by MIC. Among the tested byproducts, acetic acid, furfural, HMF, and LDPs showed significant adverse effects against XR with the IC_{50} values of 11, 2.3, 0.4, and 4 g/L, respectively and HMF had the largest XR inhibitory activity. It is noted that the content of byproducts present in the MWSHH showed insignificant inhibitory effect on XR activity (inhibition <15%). Thus, the crude MWSHH can be used as a source of xylose for the enzymatic production of xylitol. The utilization of cheap MWSHH as an alternative to more commonly utilized high-priced medium would result in a reasonable reduction in costs of XR enzyme preparation.

The final phase was performed to optimize xylitol synthesis from xylose-rich MWSHH by XR. A sequential optimization strategy based on OFAT experimental plan and statistical program was adopted to improve xylitol production. The OFAT approach was implemented to evaluate the possible optimum levels of variables that influence xylitol synthesis. The optimum values of tested variables were found to be reaction time 10 h, temperature 30 ºC, pH 7.0, xylose concentration 18.8 g/L, NADPH concentration 3.66 g/L, enzyme concentration 3% (v/v) (0.33 U/mL), and agitation rate 100 rpm. The yield of xylitol obtained under these conditions was 56.01% (w/w). The OFAT results indicated that among the seven factors examined, five factors reaction time, temperature, pH, NADPH concentration, and enzyme concentration markedly influenced the biosynthesis of xylitol from MWSHH. The effect of these variables was further examined, by a two-level FFD, to screen the variables that significantly affect xylitol synthesis. The screening design identified that reaction time, temperature, and pH are the most significant among the variables tested with the values of 12 h, 35 ºC, and 7.0, respectively. These conditions led to a xylitol yield of 71.11%. The optimization study was further continued with the CCD to develop a correlation model between the most important variables for optimum modes of operating condition with respect to xylitol yield and productivity. The validity of the quadratic models was justified, and the optimum conditions were found as reaction time for 12.25 h, temperature at 35 ºC and pH 6.5, which gave the highest xylitol production of 16.28 g/L with a yield and productivity of 86.57% and 1.33 g/L·h, respectively. It is highlighted that optimization of process conditions using sequential strategies resulted in 1.55-fold improvement in overall xylitol synthesis. Thus, the combined effects of OFAT method

and statistical design proved effective in finding the most critical variables that had a significant impact on xylitol production from MWSHH-based medium and in optimizing the process. This study is the first to report the application of the statistical experimental design to improve xylitol production from hemicellulosic hydrolysate using XR. This study innovatively developed a reaction medium to produce xylitol at a considerable level using MWS hydrolysate. It is important to mention that the use of hemicellulosic hydrolysate as a source of xylose will discourage the utilization of high priced commercial xylose and also yield a high value product, xylitol from low-cost hydrolysate of MWS.

7.3 RECOMMENDATIONS FOR FUTURE WORK

The present study has generated a lot of important information on the possibility of xylitol production from crude hemicellulosic hydrolysate by isolated XR. However, the information is still unavailable on enzyme inhibition during xylitol production and the possibility of recycling the enzyme by immobilization. Thus, further studies from different corners are required to improve enzymatic xylitol production system. The findings documented in this study will play a significant role in this respect. Hence, several recommendations are made to enhance further study in the production of xylitol from lignocellulosic hydrolysate, which are mentioned below:

(i) The main bottleneck in the bioconversion process utilizing hemicellulosic hydrolysate is the inhibitory effect of some components derived from acid hydrolysis of lignocellulosic materials (LCMs). Xylose concentration was found to be crucial in governing xylitol synthesis. The MWSHH used has low xylose concentration and gives low xylitol yield compared to that can be achieved by using commercial pure xylose. Concentrating the hydrolysate also increases the concentration of toxic substances and affects the enzyme activity. Therefore, it seems that there is a real need to overcome the problems related to reaction inhibition during enzyme-based xylitol production from concentrated hydrolysate. These problems can be solved by treatment of the hydrolysate prior to reaction or by enzymatic hydrolysis of LCMs to produce a hydrolysate free from unwanted co-products.

(ii) Immobilization of XR can be studied in future, to reduce the enzyme utilization cost by recycling the enzyme and to obtain higher yield of xylitol. Support materials can be designed for immobilization that can have dual function of binding the enzyme and the substrate as well to retain the unreacted substrate for longer time and at the same time release the product instantly.

(iii) The cost of XR preparation can also be reduced through the development of high-yield microbial strains. Thus, genetic engineering should be targeted to construct recombinant bacterial strains capable of higher XR production in simple media like lignocellulosic hydrolysate. Xylitol production cost can be reduced through the utilization of enzymatic membrane reactor in batch or continuous system as XR can be recycled and reused. Membrane technology can alternatively be used, in the batch or continuous reactor, to retain enzymes and the degradation of XR can be examined for recycling purpose.

(iv) Further research should be conducted on the scale-up study in determining the suitability of the innovative enzymatic approach in industrial applications particularly for the long term usage with respect to the production of high yield and productivity of xylitol.

(v) Further study should also be directed to the purification and recovery of xylitol from the reaction mixture via crystallization.

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APPENDIX A

CHEMICAL LIST, WORKING BUFFERS, REAGENTS AND SOLUTIONS

APPENDIX A1

Table A.1: List of chemicals used in this study

Name of chemicals	Manufacturer
Acetic acid (glacial)	Merck, Germany
Acetone	Merck, Germany
Acetonitrile	Merck, Germany
Activated charcoal	Merck, Germany
Agar	R & M Chemicals, UK
L-(+)-Arabinose	Merck, Germany
Bovine serum albumin (BSA)	Sigma, USA
Calcium oxide (powder)	Merck, Germany
Copper(II) sulfate pentahydrate	Merck, Germany
Dipotassium hydrogen phosphate	Merck, Germany
Ethanol	Merck, Germany
Ferric chloride	Sigma, USA
Folin-Ciocalteu's phenol reagent (FCR)	Merck, Germany
Furfural	Merck, Germany
D-(+)-Glucose	Sigma, USA
Glycerol	Merck, Germany
Glycine	Merck, Germany
Gum arabic	Sigma, USA
Hydrochloric acid (HCl)	Merck, Germany
5-Hydroxymethyl-2-furaldehyde (HMF)	Merck, Germany
Magnesium sulphate heptahydrate	Sigma-Aldrich, USA
2-Mercaptoethanol	Sigma-Aldrich, USA
Methanol	Merck, Germany
β-Nicotinamide adenine dinucleotide 2′-phosphate tetrasodium salt hydrate reduced form (NADPH)	Sigma, USA
Peptone	Merck, Germany
Phosphoric acid	Fisons, UK
Potassium dihydrogen phosphate	Merck, Germany
Potassium ferricyanide(III), powder	Sigma-Aldrich, USA
Potassium sodium tartrate	Sigma-Aldrich, USA
Sodium acetate	Sigma-Aldrich, USA
Sodium carbonate	Merck, Germany
sodium chloride	Sigma-Aldrich, USA
Sodium hydroxide	Merck, Germany
Sulfuric acid	Merck, Germany
Tannic acid	Sigma-Aldrich, USA
Toluene	Merck, Germany
D-(+)-Xylose (standard)	Merck, Germany
D-(+)-Xylose	Sigma, USA
Xylitol	Merck, Germany
Yeast extract	R & M Chemicals, UK

APPENDIX A2

PREPARATION OF BUFFER AND OTHER SOLUTIONS FOR XR ASSAY

1) Sodium acetate buffer, 0.1 M

   Solution A: 11.55 mL glacial acetic acid per liter (0.2 M)

   Solution B: 16.40 g sodium acetate per liter (0.2 M)

   Referring to Table A.2 for desired pH, mix the indicated volumes of solution A and B, then diluted with ultrapure water to a total volume of 200 mL.

Table A.2: Preparation of 0.1 M sodium acetate buffer

Desired pH	Solution A (mL)	Solution B (mL)
4.0	82.0	18.0
5.0	29.6	70.4

2) Potassium phosphate buffer, 0.1 M

   Solution A: 27.2 g KH_2PO_4 per liter (0.2 M)

   Solution B: 45.6 g K_2HPO_4 per liter (0.2 M)

   Referring to Table A.3 for desired pH, mix the indicated volumes of solution A and B, then diluted with ultrapure water to a total volume of 200 mL.

Table A.3: Preparation of 0.1 M potassium phosphate buffer

Desired pH	Solution A (mL)	Solution B (mL)
6.0	39.0	61.0
7.0	6.5	93.5
8.0	5.3	94.7

3) Glycine-NaOH buffer, 0.1 M

Solution A: 15.01 g glycine per liter (0.2 M)

Solution B: 8 g NaOH per liter (0.2 M)

Referring to Table A.4 for desired pH, mix the indicated volumes of solution A and B, then diluted with ultrapure water to a total volume of 200 mL.

Table A.4: Preparation of 0.1 M glycine-NaOH buffer

Desired pH	Solution A (mL)	Solution B (mL)
9.0	50.0	8.8
10.0	50.0	32.0

4) 2-Mercaptoethanol solution, 0.1 M

1.28 mL of 2-Mercaptoethanol per liter (0.1 M) in 0.1 M potassium phosphate (pH 7.0)

5) NADPH solution, 3.4 mM

2.83 g NADPH per liter in 0.1 M potassium phosphate (pH 7.0)

6) D-xylose solution, 0.5 M

75 g D-xylose per liter in ultrapure water

APPENDIX A3

PREPARATION OF REAGENTS FOR PRUSSIAN BLUE METHOD

1) 0.016 M Potassium ferricyanide solution, 500 mL

$K_3Fe(CN)_6$ salt	2.63 g
Ultrapure water	400 mL
Filtration	Whatman 4

Add ultrapure water to make 500 mL solution and store at 4 ºC until use

2) 0.02 M Ferric chloride ($FeCl_3$) solution in 0.1 N HCl, 500 mL

$FeCl_3 \cdot 6H_2O$	2.70 g
Ultrapure water	400 mL
HCl (37%)	4.18 mL
Filtration	Whatman No. 4

Add ultrapure water to make 500 mL and store at 4 ºC until use

3) 6.03 M Phosphoric acid (H_3PO_4) solution, 500 mL

H_3PO_4 (85%)	204.5 mL
Ultrapure water	295.5 mL

4) 1% Gum arabic solution, 500 mL

Gum arabic	5 g
Ultrapure water	400 mL
Boiling	30 min
Filtration	Whatman 541

Add hot ultrapure water to make 500 mL and store at 4 ºC until use

APPENDIX A4
PREPARATION OF REAGENTS FOR LOWRY METHOD

Lowry Reagent

1) Reagent A: 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) (in ultrapure water)

2) Reagent B: 0.5% copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in 1% potassium sodium tartrate ($C_4H_4KNaO_6 \cdot 4H_2O$)

3) Reagent C: Mix 50 mL of reagent A with 1 mL of reagent B (50:1) in a plastic beaker just before use.

4) Reagent D: Dilute the Folin-Ciocalteu's phenol reagent (FCR; 2N) with an equal volume of ultrapure water to make it 1N in acid prior to use.

APPENDIX B

EXPERIMENTAL DATA

Table B.1: Data of the growth profile of *Candida tropicalis* in hydrolysate medium

Incubation time (h)	OD at 600 nm	DCW (g/L)
0	0.60 ± 0.06	0.42 ± 0.05
0.25	0.60 ± 0.05	0.42 ± 0.05
0.5	0.61 ± 0.04	0.42 ± 0.05
0.75	0.63 ± 0.05	0.42 ± 0.05
1	0.63 ± 0.04	0.43 ± 0.04
1.5	0.76 ± 0.05	0.46 ± 0.04
2	0.94 ± 0.06	0.50 ± 0.05
3	1.32 ± 0.09	0.64 ± 0.05
4	1.78 ± 0.14	0.82 ± 0.07
5	2.26 ± 0.26	1.11 ± 0.13
6	2.94 ± 0.29	1.33 ± 0.13
7	3.51 ± 0.32	1.57 ± 0.16
8	4.14 ± 0.26	1.82 ± 0.16
9	4.70 ± 0.29	2.11 ± 0.16
10	5.18 ± 0.35	2.34 ± 0.16
11	5.72 ± 0.38	2.52 ± 0.20
12	6.21 ± 0.46	2.76 ± 0.21
13	6.75 ± 0.54	2.98 ± 0.24
14	7.24 ± 0.54	3.20 ± 0.24
15	7.78 ± 0.62	3.44 ± 0.27
16	8.44 ± 0.73	3.68 ± 0.32
17	9.68 ± 0.76	4.02 ± 0.33
18	11.08 ± 0.77	4.36 ± 0.32
19	11.68 ± 0.88	4.68 ± 0.34
20	11.98 ± 0.89	4.87 ± 0.39
21	11.99 ± 0.88	4.88 ± 0.38
22	11.97 ± 0.89	4.88 ± 0.39
23	11.96 ± 0.90	4.88 ± 0.40
24	11.96 ± 0.92	4.88 ± 0.39
26	11.16 ± 0.96	4.66 ± 0.39
28	10.56 ± 0.97	4.48 ± 0.41
30	10.26 ± 0.97	4.21 ± 0.41
32	10.11 ± 0.97	4.06 ± 0.41
34	10.10 ± 0.97	4.05 ± 0.42
36	10.08 ± 0.98	4.03 ± 0.43

OD = optical density, DCW = dry cell weight

Table B.2: Results of kinetic studies on xylose reduction by XR

Expt. No	Substrate conc.		Xylitol (mM)	Velocities (µM/min)		$1/[S_X]$ (mM^{-1})	$1/[S_N]$ (μM^{-1})	$1/V_X$ (min/µM)	$1/V_N$ (min/µM)
	Xylose (mM)	NADPH (µM)		V_X	V_N				
1	40	300	39.29	53.45		0.0250		0.0187 ± 0.0017	
2	80		63.99	87.06		0.0125		0.0115 ± 0.0015	
3	120		84.36	114.77		0.0083		0.0087 ± 0.0012	
4	160		93.23	126.84		0.0063		0.0079 ± 0.0009	
5	200		104.59	142.31		0.0050		0.0070 ± 0.0010	
6	240		110.51	150.35		0.0042		0.0067 ± 0.0011	
7	280		116.35	158.31		0.0036		0.0063 ± 0.0010	
8	320		119.31	162.33		0.0031		0.0062 ± 0.0009	
9	360		119.44	162.51		0.0028		0.0062 ± 0.0009	
10	400		119.84	163.04		0.0025		0.0061 ± 0.0009	
11	280	15	6.18		8.40		0.0667		0.1190 ± 0.0111
12		30	7.69		10.46		0.0333		0.0956 ± 0.0121
13		45	7.88		10.73		0.0222		0.0932 ± 0.0124
14		60	8.15		11.08		0.0167		0.0902 ± 0.0105
15		75	8.02		10.91		0.0133		0.0917 ± 0.0124
16		90	8.15		11.08		0.0111		0.0902 ± 0.0148
17		105	8.41		11.44		0.0095		0.0874 ± 0.0138
18		120	8.48		11.53		0.0083		0.0867 ± 0.0121
19		135	8.80		11.98		0.0074		0.0835 ± 0.0125
20		150	9.00		12.25		0.0067		0.0817 ± 0.0114

$[S_X]$ = xylose concentration, $[S_N]$ = NADPH concentration
V_X = initial velocity of XR for xylose, V_N = initial velocity of XR for NADPH

Table B.3: OFAT experimental plan and composition of MWS hydrolysate obtained in each run

Run	Factors levels				Concentration (g/L)						
	Temperature (ºC)	H_2SO_4 conc. (%, w/w)	Time (min)	LSR (g/g)	Xylose	Glucose	Arabinose	Acetic acid	Furfural	HMF	LDPs
1	130	2	10	8	8.28 ± 0.23	0.63 ± 0.03	1.58 ± 0.03	1.84 ± 0.07	0.11 ± 0.005	0.013 ± 0.001	0.85 ± 0.01
2	130	2	20	8	10.28 ± 0.28	1.42 ± 0.06	2.08 ± 0.04	2.78 ± 0.13	0.20 ± 0.009	0.020 ± 0.001	1.05 ± 0.02
3	130	2	30	8	12.83 ± 0.32	2.45 ± 0.11	2.29 ± 0.05	3.26 ± 0.15	0.27 ± 0.009	0.030 ± 0.001	1.25 ± 0.02
4	130	2	40	8	14.28 ± 0.33	3.22 ± 0.15	2.36 ± 0.03	3.72 ± 0.15	0.34 ± 0.017	0.040 ± 0.002	1.38 ± 0.02
5	130	2	50	8	14.59 ± 0.36	3.60 ± 0.17	2.42 ± 0.06	3.96 ± 0.16	0.39 ± 0.016	0.055 ± 0.002	1.53 ± 0.03
6	130	2	60	8	14.75 ± 0.42	3.90 ± 0.18	2.47 ± 0.12	4.10 ± 0.10	0.46 ± 0.015	0.070 ± 0.003	1.73 ± 0.03
7	130	2	70	8	14.73 ± 0.41	4.19 ± 0.18	2.44 ± 0.05	4.31 ± 0.13	0.53 ± 0.025	0.078 ± 0.001	2.00 ± 0.05
8	130	2	80	8	14.70 ± 0.40	4.50 ± 0.15	2.24 ± 0.05	4.37 ± 0.13	0.58 ± 0.025	0.088 ± 0.001	2.15 ± 0.03
9	130	2	90	8	14.62 ± 0.35	4.85 ± 0.18	2.17 ± 0.05	4.42 ± 0.13	0.60 ± 0.027	0.103 ± 0.002	2.38 ± 0.03
10	130	2	100	8	14.52 ± 0.33	5.25 ± 0.18	2.12 ± 0.06	4.44 ± 0.12	0.62 ± 0.026	0.113 ± 0.005	2.60 ± 0.03
11	130	2	110	8	14.32 ± 0.31	5.72 ± 0.20	2.08 ± 0.06	4.49 ± 0.12	0.66 ± 0.029	0.128 ± 0.003	2.87 ± 0.04
12	130	2	120	8	14.13 ± 0.22	6.22 ± 0.22	2.00 ± 0.07	4.52 ± 0.15	0.73 ± 0.031	0.138 ± 0.004	3.10 ± 0.05

Table B.3: Continued

Run	Factors levels				Concentration (g/L)						
	Temperature (°C)	H_2SO_4 conc. (%, w/w)	Time (min)	LSR (g/g)	Xylose	Glucose	Arabinose	Acetic acid	Furfural	HMF	LDPs
13	105	2	60	8	5.83 ± 0.16	1.05 ± 0.05	0.75 ± 0.02	2.67 ± 0.09	0.01 ± 0.001	0.003 ± 0.0001	0.48 ± 0.01
14	110	2	60	8	6.13 ± 0.17	1.15 ± 0.04	0.85 ± 0.03	2.75 ± 0.03	0.01 ± 0.001	0.005 ± 0.0002	0.48 ± 0.01
15	115	2	60	8	8.83 ± 0.29	2.20 ± 0.05	1.48 ± 0.03	3.04 ± 0.04	0.13 ± 0.004	0.018 ± 0.001	0.75 ± 0.02
16	120	2	60	8	12.25 ± 0.13	2.86 ± 0.10	1.93 ± 0.04	3.72 ± 0.06	0.19 ± 0.004	0.025 ± 0.001	0.95 ± 0.03
17	125	2	60	8	14.78 ± 0.30	3.35 ± 0.08	2.44 ± 0.05	3.95 ± 0.05	0.28 ± 0.007	0.053 ± 0.001	1.10 ± 0.02
18	130	2	60	8	14.75 ± 0.42	3.90 ± 0.18	2.47 ± 0.12	4.10 ± 0.10	0.46 ± 0.015	0.070 ± 0.003	1.73 ± 0.03
19	125	2	60	8	14.78 ± 0.30	3.35 ± 0.08	2.44 ± 0.05	3.95 ± 0.05	0.28 ± 0.007	0.053 ± 0.001	1.10 ± 0.02
20	125	4	60	8	17.90 ± 0.32	4.69 ± 0.07	2.59 ± 0.04	4.45 ± 0.07	0.45 ± 0.007	0.078 ± 0.001	1.53 ± 0.02
21	125	6	60	8	17.91 ± 0.24	6.07 ± 0.09	2.59 ± 0.07	4.68 ± 0.07	0.55 ± 0.007	0.088 ± 0.003	1.70 ± 0.04
22	125	8	60	8	16.28 ± 0.35	9.15 ± 0.12	2.42 ± 0.06	5.11 ± 0.06	0.72 ± 0.023	0.103 ± 0.003	1.85 ± 0.03
23	125	10	60	8	16.05 ± 0.24	10.97 ± 0.14	2.26 ± 0.05	5.48 ± 0.13	0.79 ± 0.026	0.115 ± 0.004	1.93 ± 0.04

Table B.3: Continued

Run	Factors levels				Concentration (g/L)						
	Temperature (°C)	H_2SO_4 conc. (%, w/w)	Time (min)	LSR (g/g)	Xylose	Glucose	Arabinose	Acetic acid	Furfural	HMF	LDPs
24	125	12	60	8	15.95 ± 0.23	12.40 ± 0.28	2.11 ± 0.04	5.78 ± 0.14	0.87 ± 0.022	0.120 ± 0.003	1.95 ± 0.03
25	125	4	60	8	17.90 ± 0.32	4.69 ± 0.07	2.59 ± 0.04	4.45 ± 0.07	0.45 ± 0.007	0.078 ± 0.001	1.53 ± 0.02
26	125	4	60	10	15.10 ± 0.48	3.92 ± 0.05	2.50 ± 0.07	4.11 ± 0.18	0.45 ± 0.015	0.070 ± 0.001	1.38 ± 0.03
27	125	4	60	12	12.28 ± 0.17	3.39 ± 0.05	2.47 ± 0.05	3.82 ± 0.16	0.43 ± 0.010	0.065 ± 0.001	1.33 ± 0.03
28	125	4	60	14	10.15 ± 0.12	2.99 ± 0.09	2.32 ± 0.03	3.59 ± 0.09	0.42 ± 0.008	0.060 ± 0.001	1.24 ± 0.02
29	125	4	60	16	8.28 ± 0.30	2.44 ± 0.09	2.11 ± 0.06	3.33 ± 0.10	0.39 ± 0.012	0.053 ± 0.001	1.15 ± 0.02
30	125	4	60	18	6.58 ± 0.11	2.04 ± 0.06	1.88 ± 0.05	3.04 ± 0.11	0.37 ± 0.006	0.048 ± 0.001	1.03 ± 0.02
31	125	4	60	20	4.85 ± 0.11	1.74 ± 0.06	1.46 ± 0.05	2.75 ± 0.06	0.32 ± 0.006	0.044 ± 0.001	0.98 ± 0.03

LSR = liquid to solid ratio, HMF = hydroxymethylfurfural, LDPs = lignin degradation products

Table B.4: Experimental layout and results of kinetic studies of the acid hydrolysis of MWS

Run	Factors levels				Concentration (g/L)							
					Xylose		Glucose		Furfural		Acetic acid	
	Tempera-ture (°C)	H_2SO_4 conc. (%, w/w)	Time (min)	LSR (g/g)	Expt	Pred	Expt	Pred	Expt	Pred	Expt	Pred
1	130	2	0	8	0	0	0	0	0	0	0	0
2	130	2	20	8	10.28 ± 0.28	10.05	1.42 ± 0.06	1.63	0.20 ± 0.009	0.16	2.78 ± 0.13	2.58
3	130	2	40	8	14.28 ± 0.33	14.29	3.22 ± 0.15	2.93	0.34 ± 0.017	0.30	3.72 ± 0.15	3.70
4	130	2	60	8	14.75 ± 0.42	15.40	3.90 ± 0.18	3.96	0.46 ± 0.015	0.43	4.10 ± 0.10	4.18
5	130	2	80	8	14.70 ± 0.40	15.36	4.50 ± 0.15	4.78	0.58 ± 0.025	0.55	4.37 ± 0.13	4.39
6	130	2	100	8	14.52 ± 0.33	14.81	5.25 ± 0.18	5.44	0.62 ± 0.026	0.66	4.44 ± 0.12	4.48
7	130	2	120	8	14.13 ± 0.32	14.29	6.22 ± 0.22	5.96	0.73 ± 0.031	0.76	4.52 ± 0.15	4.52
8	130	4	0	8	0	0	0	0	0	0	0	0
9	130	4	20	8	17.78 ± 0.40	17.71	2.55 ± 0.12	2.64	0.28 ± 0.013	0.35	3.75 ± 0.11	3.53
10	130	4	40	8	18.65 ± 0.58	18.19	4.50 ± 0.20	4.48	0.64 ± 0.026	0.67	4.29 ± 0.17	4.52
11	130	4	60	8	17.19 ± 0.73	16.59	5.80 ± 0.19	5.74	0.96 ± 0.044	0.94	4.53 ± 0.14	4.79
12	130	4	80	8	15.85 ± 0.67	15.22	6.70 ± 0.25	6.62	1.16 ± 0.044	1.19	4.60 ± 0.20	4.87
13	130	4	100	8	13.98 ± 0.38	13.96	7.07 ± 0.29	7.23	1.37 ± 0.058	1.40	4.65 ± 0.21	4.89
14	130	4	120	8	12.33 ± 0.56	12.80	7.70 ± 0.34	7.65	1.66 ± 0.067	1.59	4.73 ± 0.18	4.90
15	130	6	0	8	0	0	0	0	0	0	0	0
16	130	6	20	8	18.53 ± 0.52	18.24	4.21 ± 0.23	4.11	0.42 ± 0.022	0.44	4.10 ± 0.19	4.07
17	130	6	40	8	17.28 ± 0.33	17.18	6.49 ± 0.33	6.37	0.78 ± 0.033	0.81	4.79 ± 0.20	4.89
18	130	6	60	8	15.23 ± 0.33	14.92	7.51 ± 0.34	7.60	1.11 ± 0.033	1.13	4.88 ± 0.16	5.06
19	130	6	80	8	13.50 ± 0.45	13.20	8.16 ± 0.40	8.28	1.41 ± 0.042	1.39	4.96 ± 0.23	5.09
20	130	6	100	8	11.30 ± 0.48	11.67	8.51 ± 0.37	8.65	1.63 ± 0.051	1.62	4.96 ± 0.24	5.10
21	130	6	120	8	10.15 ± 0.34	10.32	8.81 ± 0.39	8.85	1.82 ± 0.051	1.81	5.03 ± 0.23	5.10

Expt = experimental, Pred = predicted

Table B.5: CCD experimental run and concentration of various compounds released during H_2SO_4 hydrolysis of MWS

CCD Run No	Concentration (g/L)						
	Xylose	**Glucose**	**Arabinose**	**Acetic acid**	**Furfural**	**HMF**	**LDPs**
1	12.21 ± 0.32	5.36 ± 0.16	2.11 ± 0.078	3.64 ± 0.13	0.58 ± 0.020	0.12 ± 0.006	1.93 ± 0.074
2	19.10 ± 0.39	5.13 ± 0.20	2.74 ± 0.103	4.36 ± 0.14	0.50 ± 0.015	0.08 ± 0.002	1.50 ± 0.038
3	17.48 ± 0.28	5.02 ± 0.20	2.73 ± 0.094	3.66 ± 0.14	0.48 ± 0.024	0.09 ± 0.005	1.48 ± 0.063
4	17.93 ± 0.46	4.69 ± 0.17	2.59 ± 0.088	4.45 ± 0.12	0.45 ± 0.014	0.08 ± 0.003	1.63 ± 0.046
5	13.09 ± 0.33	3.60 ± 0.19	2.21 ± 0.097	3.38 ± 0.14	0.48 ± 0.018	0.09 ± 0.004	1.63 ± 0.076
6	18.22 ± 0.39	4.78 ± 0.18	2.69 ± 0.086	4.26 ± 0.13	0.48 ± 0.017	0.07 ± 0.002	1.50 ± 0.032
7	17.90 ± 0.31	4.48 ± 0.14	2.57 ± 0.078	4.16 ± 0.12	0.48 ± 0.015	0.08 ± 0.003	1.60 ± 0.043
8	14.66 ± 0.33	3.35 ± 0.14	2.44 ± 0.063	3.95 ± 0.18	0.28 ± 0.012	0.05 ± 0.003	1.10 ± 0.047
9	15.97 ± 0.44	2.94 ± 0.15	2.55 ± 0.073	3.06 ± 0.15	0.40 ± 0.016	0.06 ± 0.003	1.30 ± 0.052
10	13.08 ± 0.29	4.61 ± 0.12	2.20 ± 0.092	3.25 ± 0.11	0.53 ± 0.011	0.11 ± 0.005	1.88 ± 0.078
11	16.25 ± 0.38	3.30 ± 0.14	2.58 ± 0.098	3.22 ± 0.13	0.40 ± 0.014	0.06 ± 0.003	1.30 ± 0.060
12	7.80 ± 0.32	1.67 ± 0.06	1.59 ± 0.086	3.01 ± 0.15	0.28 ± 0.011	0.04 ± 0.002	1.15 ± 0.031
13	12.65 ± 0.32	4.32 ± 0.11	1.80 ± 0.077	3.40 ± 0.13	0.53 ± 0.024	0.11 ± 0.005	1.85 ± 0.038
14	8.64 ± 0.26	1.83 ± 0.09	1.69 ± 0.068	3.77 ± 0.20	0.23 ± 0.012	0.04 ± 0.002	0.85 ± 0.034
15	11.26 ± 0.25	2.59 ± 0.08	1.99 ± 0.065	3.17 ± 0.16	0.35 ± 0.017	0.06 ± 0.003	1.13 ± 0.045
16	18.09 ± 0.56	3.74 ± 0.16	2.77 ± 0.101	3.04 ± 0.13	0.38 ± 0.012	0.07 ± 0.003	1.28 ± 0.053
17	17.23 ± 0.31	4.58 ± 0.14	2.46 ± 0.061	4.36 ± 0.17	0.48 ± 0.021	0.08 ± 0.003	1.40 ± 0.044
18	16.39 ± 0.60	6.07 ± 0.17	2.59 ± 0.093	4.68 ± 0.19	0.55 ± 0.024	0.09 ± 0.004	1.70 ± 0.061
19	18.72 ± 0.62	4.99 ± 0.19	2.88 ± 0.103	3.72 ± 0.13	0.50 ± 0.018	0.09 ± 0.004	1.68 ± 0.065
20	16.89 ± 0.26	4.55 ± 0.19	2.44 ± 0.062	4.19 ± 0.16	0.45 ± 0.013	0.08 ± 0.002	1.50 ± 0.051

CCD = central composite design, HMF = hydroxymethylfurfural, LDPs = lignin degradation products

Table B.6: OFAT design matrix with experimental values of xylitol production from MWS hydrolysate using XR

Run	Levels of factors							Xylitol conc. (g/L)	Xylitol yield ($Y_{p/s}$; %)
	Time (min)	Temperature (°C)	pH	Xylose conc. (g/L)	NADPH conc. (g/L)	Enzyme conc. (%, v/v)	Agitation (rpm)		
1	2	25	6.0	18.8	2.83	5	150	1.04	5.53 ± 0.92
2	4	25	6.0	18.8	2.83	5	150	3.39	18.03 ± 2.55
3	6	25	6.0	18.8	2.83	5	150	5.84	31.06 ± 3.88
4	8	25	6.0	18.8	2.83	5	150	7.31	38.88 ± 3.56
5	10	25	6.0	18.8	2.83	5	150	7.73	41.12 ± 3.30
6	12	25	6.0	18.8	2.83	5	150	7.73	41.12 ± 3.40
7	14	25	6.0	18.8	2.83	5	150	7.73	41.12 ± 3.09
8	16	25	6.0	18.8	2.83	5	150	7.74	41.14 ± 3.35
9	10	20	6.0	18.8	2.83	5	150	6.11	32.50 ± 3.40
10	10	25	6.0	18.8	2.83	5	150	7.73	41.12 ± 3.30
11	10	30	6.0	18.8	2.83	5	150	9.62	51.17 ± 4.31
12	10	35	6.0	18.8	2.83	5	150	9.63	51.17 ± 3.46
13	10	40	6.0	18.8	2.83	5	150	9.62	51.17 ± 4.41
14	10	50	6.0	18.8	2.83	5	150	6.98	33.13 ± 4.46
15	10	60	6.0	18.8	2.83	5	150	4.76	15.32 ± 2.12
16	10	70	6.0	18.8	2.83	5	150	2.21	2.76 ± 0.39
17	10	30	4.0	18.8	2.83	5	150	0.84	4.47 ± 0.85
18	10	30	5.0	18.8	2.83	5	150	5.17	27.50 ± 2.71
19	10	30	6.0	18.8	2.83	5	150	9.62	51.17 ± 4.31
20	10	30	7.0	18.8	2.83	5	150	9.71	51.65 ± 4.41
21	10	30	8.0	18.8	2.83	5	150	5.15	27.39 ± 3.08
22	10	30	9.0	18.8	2.83	5	150	1.83	9.73 ± 1.75
23	10	30	7.0	9.4	2.83	5	150	3.45	36.70 ± 5.43

Table B.6: Continued

Run	Levels of factors							Xylitol conc. (g/L)	Xylitol yield ($Y_{p/s}$; %)
	Time (min)	Temperature (°C)	pH	Xylose conc. (g/L)	NADPH conc. (g/L)	Enzyme conc. (%, v/v)	Agitation (rpm)		
24	10	30	7.0	14.1	2.83	5	150	5.81	41.21 ± 3.90
25	10	30	7.0	18.8	2.83	5	150	9.71	51.65 ± 4.41
26	10	30	7.0	23.5	2.83	5	150	9.66	41.11 ± 3.28
27	10	30	7.0	28.2	2.83	5	150	8.18	29.01 ± 2.70
28	10	30	7.0	32.9	2.83	5	150	6.22	18.91 ± 2.00
29	10	30	7.0	37.6	2.83	5	150	5.12	13.62 ± 1.54
30	10	30	7.0	18.8	1.17	5	150	3.56	18.94 ± 1.91
31	10	30	7.0	18.8	2.00	5	150	7.33	38.99 ± 4.15
32	10	30	7.0	18.8	2.83	5	150	9.71	51.65 ± 3.67
33	10	30	7.0	18.8	3.66	5	150	10.12	53.83 ± 3.94
34	10	30	7.0	18.8	4.49	5	150	10.12	53.83 ± 3.98
35	10	30	7.0	18.8	5.32	5	150	10.12	53.83 ± 4.04
36	10	30	7.0	18.8	3.66	2	150	10.12	53.83 ± 3.94
37	10	30	7.0	18.8	3.66	3	150	10.53	56.01 ± 5.11
38	10	30	7.0	18.8	3.66	4	150	10.53	56.01 ± 4.89
39	10	30	7.0	18.8	3.66	5	150	9.81	52.18 ± 4.41
40	10	30	7.0	18.8	3.66	6	150	9.53	50.69 ± 4.31
41	10	30	7.0	18.8	3.66	3	50	8.46	45.00 ± 3.24
42	10	30	7.0	18.8	3.66	3	75	10.21	54.31 ± 4.63
43	10	30	7.0	18.8	3.66	3	100	10.53	56.01 ± 4.20
44	10	30	7.0	18.8	3.66	3	125	10.53	56.01 ± 4.31
45	10	30	7.0	18.8	3.66	3	150	10.53	56.01 ± 4.57

Xylitol yield ($Y_{p/s,}$ %) = [xylitol produced (g/L)/ xylose consumed (g/L)] × 100

APPENDIX C

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS RESULTED FROM THIS STUDY

Referred Journals

1. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Utilization of *Meranti* wood sawdust as a raw material to produce xylose by acid hydrolysis. *Biomass and Bioenergy* (Submitted).

2. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Xylose extraction from wood sawdust by acid hydrolysis: One-factor-at-a-time study. *American Journal of Engineering and Applied Sciences* (Accepted, Scopus Indexed).

3. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Processes for the production of xylitol – a review. *Food Reviews International*. DOI: 10.1080/87559129.2012. 714434 (In press, Scopus/ISI Indexed, **IF = 1.45**).

4. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Design of process parameters for the production of xylose from wood sawdust. *Chemical Engineering Research and Design*. **90**(9): 1307–1312 (Scopus/ISI Indexed, **IF = 1.97**).

5. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Kinetic studies on acid hydrolysis of *Meranti* wood sawdust for xylose production. *Chemical Engineering Science*. **71**: 431–437 (Scopus/ISI Indexed, **IF = 2.43**).

6. Rafiqul, I.S.M. and Sakinah, A.M.M. 2012. Bioproduction of xylitol by enzyme technology and future prospects – a perspective. *International Food Research Journal*. **19**(2): 405–408 (Scopus Indexed).

7. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Kinetic modeling of the acid hydrolysis of wood sawdust. *International Journal of Chemical and Environmental Engineering*. **2**(5): 333–337.

Conferences/Proceedings

8. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Utilization possibilities of *Meranti* mood sawdust as a raw material to produce xylose by acid hydrolysis in Malaysia. *ICCEIB-SOMChE 2011*, 28th November to 1st December 2011, Kuantan, Pahang, Malaysia.

9. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Determination of optimum range of process variables using one-factor-at-a-time method for xylose production from wood sawdust. *Proceedings of 1st International Conference and Exhibition of Women Engineer (ICEWE'2011)*, November 20–22, Gambang Resort City, Kuantan, Malaysia, pp. 21–25.

10. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Optimization of xylose recovery from wood sawdust by acid hydrolysis. *2011 World Congress on Engineering and Technology (CET' 2011)*, October 28–30, **Shanghai, China**, pp. 261–264.

11. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Kinetic modeling of the acid hydrolysis of wood sawdust. *Proceedings of International Renewable Energy and Environment Conference (IREEC' 2011)*, June 24–26, Kuala Lumpur, Malaysia, pp. 1–5.

12. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Enzymatic production of xylitol and future prospects. *Proceedings of the 2nd International Conference on Biotechnology Engineering (ICBioE'11)*, May 17–19, Kuala Lumpur, Malaysia, pp. 176–178.

13. Rafiqul, I.S.M. and Sakinah, A.M.M. 2011. Optimization studies on *Meranti* wood sawdust acid hydrolysis for xylose production. *Post Graduates Poster competition' 2011*, April 7–9 (Poster presentation), Astaka, Universiti Malaysia Pahang, Malaysia.