PROCESS OPTIMIZATION FOR XYLITOL PURIFICATION USING LIQUID-LIQUID BATCH EXTRACTION: EFFECT OF VOLUME RATIOS AND NUMBER OF STAGES

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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature :
Name of main supervisor : PROF. DR. MIMI SAKINAH
Position : PROFESSOR
Date : 20 JANUARY 2015

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
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Dedication

I would like to dedicate my thesis to my beloved supervisor, friends and family who supported me each step of the way.

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ABSTRACT

Xylitol is a five-carbon sugar alcohol that has many significant applications in the food, and pharmaceutical industries owing to its unique properties. Xylitol has proven its core value in reducing tooth decay and as sugar substitute for diabetes patient. In general, purification steps for production of fine product are of great interest in process engineering due to the complexity of the separation processes which leads to the major costing in production. Liquid-liquid extraction is a simple, time and energy saving separation method that employed to separate xylitol and impurities. Objective of this research is to optimize the conditions for purification of xylitol with ethyl acetate as solvent using response surface methodology in batch solvent extraction. Experiment design were done with one factor at time method to screen the significance of various factors, then RSM analysis was performed and experiments was conducted based on the suggested models. All the experiments were done in three replications to minimize systematic and instrument errors. OFAT results had shown that the time of extraction is not significant but the optimum conditions for volume ratio of sample to solvent is 1:5, and the number of multiple staging are 5 stages. Henceforth in RSM analysis, first factor was set to be the volume ratio of sample to solvent ranging from 1:2 to 1:6 and second factor was set to be the number of staging ranging from 2 to 6 stages. RSM analysis results that the overall model is valid, with correlation p-value of 0.002 (<0.05) with both two factors and factors' squared proven to be significant to the response. The lack of fit value of 0.0003 for the model is significant. Feasibility study which is the xylitol extraction optimization using above suggested model by RSM produced from Meranti wood is validated. Results obtained from this research have demonstrated that both volume ratio and number of stages have significant effects in extraction process of xylitol purification and through careful optimization the downstream processing of commercial xylitol could be more effective.

ABSTRAK

Xilitol merupakan sejenis pemanis yang mempunyai 5 karbon dan didapati mempunyai banyak aplikasi dalam industri makanan and farmasi. Xilitol telah dibuktikan bahawa ia mampu mengurangkan kerosakan gigi and digunakan secara am sebagai penganti gula dalam pasaran terutamanya untuk penyakit kencing manis. Secara umum, penghasilan dan proses meningkatkan ketulenan sesuatu bahan domestik merupakan masalah yang utama bagi para jurutera. Kerumitan proses akan membawa kepada kenaikan kos penghasilan. Pengekstrakan cecair merupakan cara yang mudah, jimat masa dan tenaga untuk memisahkan xilitol dan bahan bio-kimia yang tidak dikehendaki. Tujuan kajian ini adalah untuk mengoptimumkan faktor-faktor yang akan mempengaruhi ketulinan xilitol selepas proses pengekstrakan. Xilitol diekstrak dengan menggunakan etil asetat. Semua eksperimen telah dijalankan atas tiga replikasi untuk mengurangkan ralat sistematik dan instrumen. Keputusan OFAT telah menunjukkan bahawa masa pengekstrakan adalah tidak signifikan. Keputusan keadaan optimum untuk nisbah isipadu sampel dan pelarut adalah 1 : 5 , manakala jumlah pelbagai peringkat adalah 5. Julat faktor pertama iaitu nisbah isipadu sampel dan pelarut adalah di antara 1 : 2 hingga 1 : 6 dan julat bagi faktor kedua adalah jumlah peringkat iaitu antara 2 hingga 6 peringkat dimasukkan ke dalam analisis RSM. Daripada keputusan yang terhasil, model keseluruhan adalah signifikan, dengan korelasi nilai-p ialah 0.002 (< 0.05) serta kedua-dua dua faktor dan faktor-faktor ' kuasa dua terbukti signifikan kepada respon . Kajian kebolehlaksanaanpengekstrakan xilitol daripada kayu Meranti menggunakan persamaan daripada keputusan RSM telah dilaksanakan dan ianya adalah sah. Keputusan yang diperolehi daripada kajian ini telah menunjukkan bahawa kedua-dua nisbah jumlah dan bilangan peringkat mempunyai kesan yang penting dalam proses pengekstrakan penulinan xilitol dan pengoptimuman yang teliti perlu diaplikasikan dalam pemprosesan hiliran xilitol secara komersial supaya ketulinan xilitol menjadi lebih berkesan..

1 INTRODUCTION

1.1 Background of study

Sweeteners play an important part of the global food and beverage industry and its production require wide range of chemical process technologies owing to the diverse sources and uses. The Polyols sweetener industry is currently in a rapid growth due to the increasing consumer demand for sugar-free and reduced calorie products. The sweeteners that contributes to the surging of this genre of food industry are the sugar alcohols such as xylitol, sorbitol, mannitol ,and maltitol.

Xylitol is a five-carbon polyol with a sweetness similar to sucrose. At 10% solids (w/w) xylitol is isosweet to sucrose. (Munton and Birch 1985). Therefore xylitol is widely manufactured in artificial sugar industry.Xylitol is not only a sugar-free sweetener, but also has unique properties that find applications in pharmaceutical, medical, and in domestic usage. (Gurgel *et al.*, 1995). It is non-cariogenic, shown to be valuable in prevention of dental caries because it is not an effective substrate for plaque bacteria and from various clinical studies ,xylitol caries prevention rates is recorded at least 80 to 85%, compared with the sucrose-using groups. (Makinen et al.,2000)

In addition, xylitol is diabetic tolerance and appears to be a promising agent in prevention of acute otitis media when administered in chewing gum. (Uhari & Tapiainen et al., 2000), Xylitol also prevents osteoporosis and lung infections. (Zabner, 2004) Diabetics and dental caries can be overcome by the substitution of xylitol in sugar contained products. Xylitol can be naturally found in some fruits and vegetables, but extraction of xylitol from these sources is economically not feasible because of the low concentrations present (Saha, 1997).

In industrial scale, xylitol is produced mainly by chemical process that involves the catalytic dehydrogenation of D-xylose that present in lignocellulosic hydrolyzates and the solution produced requires subsequent high-cost purification and separation process

to obtain high purity xylitol. (Gurgel et al. 1995) . Nontheless it can be produced via biotechnological method i.e bioconversion of xylose-to-xylitol utilizing microorganisms or enzymes which appears to be economically feasible. In the past decade, yeasts have been studied most widely for the production of xylitol, with extensive emphasis on the genera *Candida* (Barbosa et al., 1988) and *Debaryomyce.* (Converti et al., 2002)

Somehow, the yield and productivity are not promising and breakingthrough purification methods besides chromatographic separation that commonly applied to the separation and recovery of the product. Moreover, the recovery and purification of the product exists as a very complicated step in industrial fermentative processes because of the low product concentration and the complex composition of the fermentation broth as in fermentation processes there are different impurities,such as proteins and carbohydrates, introduced for microbial growth. (Granström & Leisola, 2013)

In general, different separation methods had been utilized and can be used in accordance to the specify needs of the manufacturer, mostly required balancing between processing cost and purity of end product. According to Mussatto et al., ethyl acetate is a promising extractor agent for xylitol purification from the fermented broth, hence is chosen to extract commercial xylitol solution. In present, less focus on LLE compare to membrane separation due to its lower efficiency but LLE is simple, cheap and fast to perform for undergraduate project.

1.2 Motivation

Oral cavity and diabetic is a major health issue globally. Conventional sugars are the main contributor. Xylitol is an artificial sweetener with good taste and approximate 67% reduced calories than conventional sugar that proven to be alternatives for glucose and sucrose. (Heikkila *et al*., 1992).

For daily diets consuming, xylitol does not promote tooth decay and have similar characteristic to both sucrose and dextrose sugars e.g. fructose. (Scheinin et al., 1976) It is not a carbohydrate but a sugar alcohol that can be obtained from the reduction of glucose, changing the aldehyde group to hydroxyl group or by microbial conversion from xylose.

Caries is caused by the dissolution of the teeth by acid produced through metabolism of dietary carbohydrates by oral bacteria. Researchers found out that two main oral bacteria involved in caries formation are of mutans streptococci and lactobacilli. (Aas et al.,2008) These oral bacteria utilized 6-carbon sugar to metabolise, sucrose and dextrose sugars are 6-carbon sugars hence inevitably impact catiogenic effects towards tooth. However, xylitol is a 5-carbon sugar that is non-fermentable by bacteria and therefore cannot act as bacteria energy reservoir, simply thus it has extra anticariogenic effects contributable to against microbial activity, simulation of saliva leading in increased buffer activity and pH increment, and remineralization enchancement. (Trahan,1995) Usage of xylitol for prolonged period is strongly believed that can reduce the caries and ever since Turku Sugar Studies back in year 1975, several laboratory studies had been carried out consistently (Makinen K.K.,2000)

Another dominant issue caused by high usage of conventional sugars in diet is diabetes.Diabetes is a group of metabolic diseases caused by the insulin inefficiency which results in high blood glucose concentration. Observations concluded that following the ingestion of xylitol, the blood glucose and insulin response are remarkedly lower than glucose or sucrose ingestion hence is a suitable sweetener for use in diabetics controlled diets. (Lyengar et al.,1985)

1.6 Scopes of Study

This research study will be done using liquid extraction with separation funnel and ethyl acetate as solvent.

i. Three main parameters will be monitored in the separation process experiment to identify the most influential factors that affects the composition of xylitol at the extracted layers using Central Composite Design in Design Expert 7.0 software, which are : volume ratio of xylitol solution to ethyl acetate, extraction stages, and the time of extraction process.

ii. The range of significance of all the parameters will be determined by one variable at time (OFAT).

iii. The optimization of the correlation of all parameters will be done using Response Surface Methodology (RSM) in Design Expert 7.0.

iv. The xylitol purities before extraction and after extraction will be analyze using High Performance Liquid Chromatography (HPLC) by comparing the RI values with the standard.

1.7 Organisation of this thesis

Chapter 1 comprises of the introduction part of this thesis which discussed on the background of the xylitol and of this study. The current issues encounter by industrial on xylitol downstream processing and its potential solutions were discuss briefly and lead to the motivation of this research study. Next, problem statement, research questions and scopes of study were clearly listed lastly the specific objectives to achieve for this research were also included.

Chapter 2 was the review part of this thesis supported by citated literature. Firstly, the history and the properties of the xylitol that needed to be mastered were presented. Then the importance of xylitol i.e. its applications were briefly discussed. Review on upstream production of xylitol were disserted. Downstream process for xylitol purification were reviewed from different previous studies and summarization of upstream and downstream were discussed in table form. Then the literature review for the process i.e. liquid-liquid extraction, analysis i.e. HPLC and statistic and optimization i.e. RSM were included in this chapter.

Chapter 3 give insight for the methodology part in this thesis comprises of chemical used in this research follow by the experimental flowchart and the study operational framework diagram. Next, the liquid-liquid extraction set up were explained in detailed. Standard and sample preparation were explained associated with standard curve generated. The experimental design of utilizing OFAT method were detailed out in sequence and the procedure to perform statistical analysis and optimization of factors affecting the response were explained. Lastly the procedure on analysis using HPLC and the XR production methodology were written correspondingly.

In Chapter 4, results from the commercial xylitol extraction were tabulated. Results from OFAT were presented in graphical manners and discussion on the trend and justifications were made appropriately. Next, result from RSM analysis divided into two part: statistical analysis part to check on experimental validity and optimization part, to check on the interaction and degree of affects to the response were tabulated with diagrams and result generated from Design Expert software. Lastly, the result for feasibility study and fermentation of xylose using XR enzyme were presented. Discussions were made on every result collected from this experimental study.

Chapter 5 were the conclusion of this research to ensure that the research objectives were met and several recommendations were given for future use.

2 LITERATURE REVIEW

2.1 Overview of the project

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2.2 History of xylitol

In September, 1890, the German chemist expert Emil Herman Fischer and his assistant, Rudolf Stahel, separated a new compound from beech chips which was named Xylit, the German word for xylitol (Fischer and Stahel, 1891). Later, in 1902, owing to his outstanding chemical accomplishments, Dr. Fischer was awarded the Nobel Prize in chemistry. Almost simultaneously with Fischer, the French chemist M.G. Bertrand had managed to isolate xylitol syrup in processing wheat and oat straw (Bertrand, 1891).

After five decades, xylitol was first found in low concentration in some plants and was widely used during World War II as sugar substitute due to global food shortage. During the 1950s, Dr. Oscar Touster's work has changed the xylitol value remarkably. He and his co-workers had concluded that xylitol is formed in the human body and its metabolism is associated with pentosuria from investigations on L-xylulose. Dr. Touster reasoned that essential pentosuria involved the accumulation and excretion of a metabolite which is readily disposed of in normal, but not in pentosuric individuals. Eventually, the product was isolated and characterized as xylitol (Touster and Shaw, 1962).

Later in year 1962 a biochemical pathway involving xylitol was discovered in mammalian tissue gave rise to the intensive studies on xylitol as a natural sweetener. In 1970 the first study on the effects of xylitol on dental plaque was started in Turku, Finland. Its clinical applications were then confirmed after publication of Turku studies in 1975 (Scheinin and Makinen, 1975).

The first commercial xylitol chewing gums (XyliFresh) were launched in Finland and in the United States on the same year. Nowadays, variety of products manufactured from xylitol, scientists investigate more potential health benefits, while engineers develop more efficient and cheaper production methods.

2.3 Properties of xylitol

Xylitol (Figure 2.1.) is a five-carbon polyol with sweetness similar to sucrose and unique chemical and physical properties contribute to its increasing growth demand.

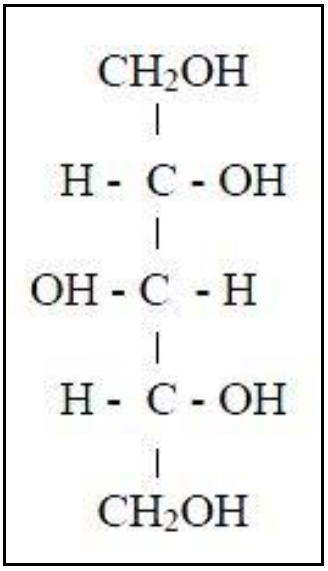


Figure 2.1 : Chemical structure of xylitol

There are many sugar polyol in the artificial sweetener market e.g. sorbitol, mannitol, erythritol and xylitol etc. Sweetness remains as the major factor for the appealing of artificial sugar production to ensure that end consumer acceptance of product taste and quality is secured. Among all, xylitol has found out to be the sweetest alternative sugar-free product with sweetness similar to sucrose (Figure 2.2). . At 10% solids (w/w) xylitol is isosweet to sucrose (Munton and Birch 1985).

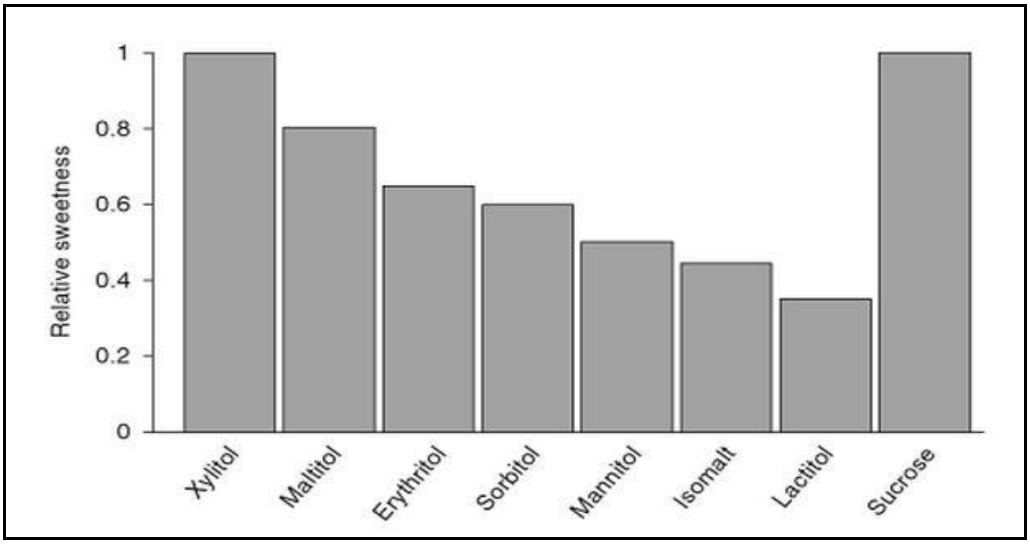


Figure 2.2 : Relative sweetness of Polyols (Moskowitz, 1971)

Among polyol, xylitol is an imperative sugar substitute with interesting physical and chemical properties which make it a high value compound for pharmaceutical, odonatological and food industries. (Table 2.1)

Other than that, xylitol also widely used in sugar-free hard coating applications due to combination of first, its excellent solubility (Aminoff et.al.,1978) which allows the production of high solids and supersaturated coating solutions and second, easily control crystallization process parameters in stable forms, and third, about 30-60% faster processing speed compare to others polyols. Chewing gum pellets are of the major example of this application.

In non-food sectors, xylitol has also found extensive use particularly in the pharmaceutical and cosmetic industries. In pharmaceutical products, xylitol is used as a sugar-free sweetener, an inert excipient and as parenteral energy source. In cosmetics, xylitol is extensively used as an effective humectant and skin-moisturising agent.
(Amaral & Camilo et al., 2011)

2.5.2 Microbial production

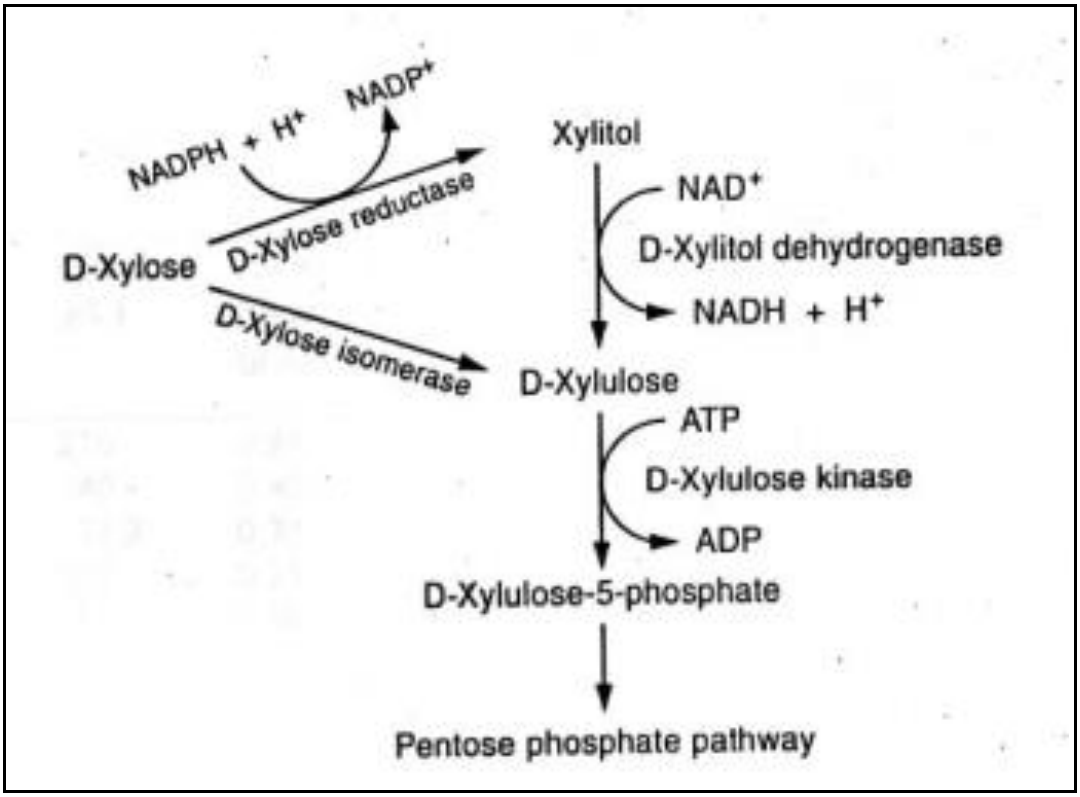


Figure 2.6 **:** Pathway for microbial xylose utilization (Saha, 1997)

There are drawbacks for the xylitol production based on catalytic hydrogenation of xylose derived from hydrolysates of hemi cellulosic rich materials; a high production cost process and requires extensive xylose purification steps (Liaw & Chen et al., 2008). Henceforth extensive research has been conducted based on the production of xylitol by fermentation using xylose present in hydrolysates derived from agro-industrial lignocellulosic residues that available in abundant, which do provide cheaper alternative in manufacturing of xylitol.

Candida yeasts have been studied extensively with regards to their biotechnological application in the production of xylitol, that xylose fermenting yeasts reduces xylose to xylitol by the NAD (P) H-dependent xylose reductase (XR) (Rafiqul and Sakinah, 2012). From the screening of different xylose-assimilating yeast, C. tropicalis was found the best xylitol producer. A volumetric productivity of 5.7 g xylitol L-1 h-1 was

achieved with 69 % yield from D-xylose on a mineral medium with a modified repeated fed batch production method. (Granström, 2002)

The production of xylitol through the fermentation process is somehow limited by certain factors, such as precise control of culture conditions, expensive nutrients, huge water consumption, and the type of process and as a consequent its viability depends on the optimization of these factors. (Sampaio et al., 2006)

2.5.3 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 catalyses the first step of D-xylose metabolism by reducing xylose to xylitol with the concomitant oxidation of NADPH to NADP+ (Ronzon et al., 2012; Woodyer et al., 2005 and Zhao et al., 2009). XR has potential biotechnological application at least in two areas: xylose ferementation for bioethanol production and conversion of xylose to xylitol (Rawat and Rao, 1996). To exploit its maximal potency on the conversion of xylose to xylitol, it is necessary to isolate and characterize XR from potential xylose-fermenting yeast. Lastly, the functional properties of XR enzyme must be known in detail to understand the relative roles of it in xylose conversion.

2.6 Xylitol purification methods

The characteristics of the xylitol molecule are important in understanding methods of recovery. These properties are the factor taking into consideration in choosing purification process.

2.6.1 *Xylitol Recovery from Fermentation Broth*

Knowing the characteristics of the xylitol molecule is critical to understanding methods of recovery. The size of the xylitol molecule has been investigated and was found to be about 0.96-0.99 nm in length and 0.3-0.33 nm maximum radius (Kiyosawa, 1991).

Physical and chemical properties of xylitol that are critical for separation from fermentation media are given in Table Nabor. The impurities found in the xylitol fermentation broth have a range of molecular sizes. Most of these impurities are residual nutrients from the fermentation and include yeast extract, polypeptides, sugars, sugar alcohols, and inorganic salts. The recovery of dilute concentrations of xylitol from such a complex mixture is a major challenge, which may explain why published literature shows only limited research in xylitol recovery from fermentation broths. Yeast extract, an impurity in the broth is composed of amino acids, peptides, oligopeptides and proteins

Table 2.5 : Composition of yeast extract

Fraction	**% of total yeast extract**	**Molecular weight**
Free amino acids	35-40	N/A
Peptides	10-15	<600
Oligopeptides	40-45	2000-3000
Other Oligopeptides and Proteins	2-5	3000-100,000

Following are the key purification review in brief, generally there are five methods depending on downstream operations in recovering xylitol derived from biomass i.e. ion-exchange, solvent extraction, precipitation, vacuum concentration following crystallization, and membrane separation.

2.6.2 Ion-exchange resins

These methods for xylitol recovery include ion-exchange resins, activated carbon, and chromatography. Both anion and cation exchange resins are used to purify xylitol from sugar cane bagasse hydrolysate fermentation broth. However, xylitol had affinity for strong cation-exchange resin (Amberlite 200C) and weak anion-exchange resin (Amberlite 94S), which caused 40-55% loss of product as the xylitol adhered to the surface of the resin. The fermentation broth was also pre-treated with activated carbon, which aim to remove both colour and proteins. The fermentation broth was added with 200 g/L activated carbon at 80 °C, pH 6 for 1 hour and successfully removed undesired impurities however about 20% of the xylitol absorbed. The solution was then filtered, concentrated and crystallized. Unfortunately, crystal recovery was very difficult because the solution was coloured and viscous. It took almost six weeks at –15 °C to crystallize the xylitol which is time consuming and costly. (Gurgel et al., 1995)

2.6.3 Solvent Extraction

The extraction of the undesired impurities from the xylitol solution was carried out using ethyl acetate, or chloroform in different volume ratio of xylitol-to-solvent. Maximum clarification of the broth was obtained with ethyl acetate having 48.07 g/l xylitol in aqueous phase and 17.01 g/l xylitol in organic phase. Extraction of the undesirable impurities was carried out using ethyl acetate, chloroform or dichloromethane. Removal of coloured substances by ethyl acetate has been observed. The importance of using solvents for hydrolysate purification via solvent extraction has shown yielding a phenolic-rich extract, which responsible in discoloration of the hydrolysate which ease the crystallization of pure xylitol in later stage (Gonzalez & Cruz et al., 2004). Somehow, liquid–liquid extractions have a tendency to form
Emulsion, hence slowing the clarification process.

2.6.4 Precipitation

High sugars and xylitol losses were observed using the precipitation method to recover xylitol. Tetrahydrofuran (THF) was the less selective solvent and promoted the highest xylitol loss. The low THF selectivity was probably a consequence of its lower polarity that became the xylitol little soluble in the solution, causing thus its precipitation. Among all the evaluated solvents acetone has the highest selectivity, causing only a 10.7% xylitol loss. (Mussatto & Santos et al., 2005). The acetone was more selective than ethanol because ethanol makes strong hydrogen bounds with xylitol while the bounds between the acetone carbonyl group and the xylitol are much weaker than the hydrogen bounds formed by ethanol (Solomons & Fryhle et al., 2010). Despite the high selectivity, acetone did not promote any clarification of the medium, and the THF was the unique solvent able to clarify the fermented broth. Lignin is partly soluble in THF, and a large quantity of it precipitates with this solvent. As lignin is basically composed of phenolic compounds, its removal consequently promotes a clarification of medium (Cathala et al., 2003).

2.6.5 Vacuum concentration and crystallization

Xylitol was purified via activated charcoal pre-treatment followed by vacuum concentration and crystallization method. In this method, before recovery, the initial xylitol concentration in the synthetic xylose fermented broth was 66.78 g/l which after recovery by activated charcoal treatment resulted in 63.31 g/l of xylitol. Thus, there was a loss of only 3.47 g/l of xylitol, which was adsorbed to charcoal. The filtered fermented broth was concentrated for ten times through rotary evaporator and commercial xylitol was added (1 g/l) for nucleation seeding. Incubation was at −20 °C for 3–4 days to initiate xylitol crystal formation. Yield of 39.33% xylitol was achieved when 1.306 g of xylitol crystals were obtained from 50 ml of the fermented broth (theoretical yield of 3.32 g xylitol from 50 ml). (Misra & Gupta et al., 2011)

2.7 Summary

Table 2.7 : Summary of xylitol production method

Method	Raw Material	Hydrogenation agent	$Y_{P/S}$ (%)	Q_p	Advantage	Disadvantage	Reference
Chemical	Xylan-containing materials	H2/Ni	50-60	n.a.	It provides a highly purifired xylose that ensures hydrogenation.	Laborious and cost/energy intensive Requirement of high temperature and pressure Low efficiency process	(Melaja & Hamalainen, 1977; Nigam and Singh 1995 and *Parajo et al*., 1998a)
Microbial	D-xylose	Yeast	65-85	2.67-12	Cost effective Low energy consumption	Time-consuming Huge water consumption Cell-recycling limitation	(Parajo *et al*., 1998a)
Enzymatic	D-xylose	XR from yeast	96	2.8-3.33	No cell recycling limitation Eco-environmentally than microbial processes High yield and productivity Easy recovery of xylitol	Cost of XR preparation	(Neuhauser *et al*., 1998)

Table 2.8 : Summary of xylitol purification method

Method	Conditions	Advantage & disadvantage	Reference
Anion and Cation exchange resins	20g activated carbon added to 100ml fermented broth at 80°C ,pH 6.0 for 1hour using strong cation-exchange resin and weak anion-exchange resin.	40-55% product loss because the xylitol adhered to the surface of the resins.	(Gurgel et al.,1995)
Liquid-liquid extraction	Neutralized hydrolysates and solvents were contacted in 250 ml baffled Erlenmeyer flasks with orbital shaking (300 rpm) at constant temperature (range 10–40°C). pH of hydrolysates was adjusted to 3 or 6.5	Maximum clarification with ethyl acetate having 48.07 g/l xylitol in aqueous phase and 17.01 g/l xylitol in organic phase.	(Cruz and Domingue z et al., 2004)
Precipitation	Filtered fermented broth was mixed with each of ethanol, acetone or tetrahydrofuran (THF) in 1:1 ratio (v/v), stirred and rest for 5 min. Solid separate via vacuum filtration and the supernatant was analysed.	Highest selectivity with acetone, promoting only a 10.7% xylitol loss. While THF able to clarify the broth.	(Mussatto & Santos et al., 2005)
Membrane Technology	10,000 MWCO HG19 polysulfone membrane Flux at 883 L/day.m^2 Pressure at 1.4 MPa	90.8% purity of xylitol obtained.	(Richard Peter Affleck ,2000)
Vacuum concentration plus crystallization (with activated carbon pretreat)	The filtered fermented broth was concentrated via rotary evaporator and undergoes seeding. Incubation was at −20 °C for 3–4 days.	Observed yield of 39.33% xylitol was achieved. Pre-treat is necessary to increase separation efficiency	(Misra & Gupta et al., 2011)

2.7.1 Justification on Table 2.7 (Enzymatic as production method)

According to Neuhauser *et al*., 1998, XR enzyme enzymatic reaction from xylose promote high yield and productivity which is important for the feasibility study of this research. High content of xylitol is needed. Easy recovery of xylitol also was the main attribute that contributed to the selection of enzymatic method to produce xylitol during feasibility study.

2.7.2 Justification on Table 2.8 (LLE as purification method)

According to Mussatto et al., ethyl acetate promising extractor agent for xylitol purification from the fermented broth, hence is chosen to extract commercial xylitol solution. In present, less focus on LLE compare to membrane but LLE is simple, cheap and fast to perform. Extensive experiments and chemicals are needed to conduct within short timeframe for undergraduate.

2.8 Liquid-liquid extraction

Liquid-liquid extraction (also called solvent extraction) was initially utilized in the petroleum industry beginning in the 1930's. It has since been utilized in numerous applications including petroleum, hydrometallurgical, pharmaceutical, and nuclear industries. Liquid-liquid extraction describes a method for separating components of a solution by utilizing an unequal distribution of the components between two immiscible liquid phases. In most cases, this process is carried out by intimately mixing the two immiscible phases, allowing for the selective transfer of solute(s) from one phase to the other, then allowing the two phases to separate. Typically, one phase will be an aqueous solution, usually containing the components to be separated, and the other phase will be an organic solvent, which has a high affinity for some specific components of the solution. The process is reversible by contacting the solvent loaded with solute(s) with

another immiscible phase that has a higher affinity for the solute than the organic phase.

The transfer of solute from one phase into the solvent phase is referred to as extraction and the transfer of the solute from the solvent back to the second (aqueous) phase is referred to as back-extraction or stripping. The two immiscible fluids must be capable of rapidly separating after being mixed together, and this is primarily a function of the difference in densities between the two phases. While limited mass transfer can be completed in a single, batch equilibrium contact of the two phases, one of the primary advantages of liquid-liquid extraction processes is the ability to operate in a continuous, multistage countercurrent mode.

2.8.1 Distribution Coefficient

In the typical example of liquid/liquid extraction, the product was a fairly large organic molecule which is not very soluble in water. On the other hand, if the product were a lower molecular weight or "small" molecule, it might be at least partially water-soluble. Therefore, it might not completely transfer into the organic layer, but also partially dissolve in the aqueous layer. For water-soluble organic materials, such as acetic acid or sugar, most of the solute will reside in the water phase. A quantitative measure of the how an organic compound will distribute between aqueous and organic phases is called the distribution coefficient

K =solubility of organic (g/100 mL)/solubility of water (g/100 mL)

$$K = \frac{[HA_{organic}]2}{[HA_{aqueous}]} \qquad \text{(eq. 2.1)}$$

The constant K is essentially the ratio of the concentrations of the solute in the two different solvents once the system reaches equilibrium. At equilibrium the molecules naturally distribute themselves in the solvent where they are more soluble. Inorganic and water soluble materials will stay in the water layer and more organic molecules will

remain in the organic layer. By using the correct solvent system, a molecule can be specifically selected and extracted from another solvent.

Since the distribution coefficient is a ratio, unless K is very large, not all of a solute will reside in the organic layer in a single extraction. Usually two, three, or four extractions of the aqueous layer with an organic solvent are carried out in sequence in order to remove as much of the desired product from the aqueous layer as possible. The effectiveness of multiple small volume extractions versus one large volume extraction was demonstrated. Say that one extraction can recover 90% of the compound. A second extraction with the same solvent was being able to pull out 90% of the remaining material. Effectively 99% of the compound was recovered with two extractions. One large extraction would have only obtained the initial 90%. Many smaller extractions are more efficient than one large extraction. This phenomenon can be proved mathematically, but in short follows the equation:

Fraction extracted into

$$\text{fraction extracted into B} = \left(\frac{1}{1 + \frac{V_B}{V_A n K}} \right)^n \qquad \text{(eq. 2.2)}$$

This equation provides the fraction of material extracted by solvent B where n is the Number of extractions performed, K is the distribution coefficient, VA is the volume of solvent A and VB is the volume of solvent B.

2.9 Analytical review (HPLC)

High-performance liquid chromatography (HPLC) is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbent, leading to the separation of the sample components. The active component of the column, the sorbent, is typically a granular material made of solid particles e.g. silica, polymers, etc., 2–50 micrometres in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the sorbent particles. The pressurized liquid is typically a mixture of solvents e.g. water, acetonitrile and/or methanol and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and sorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination. HPLC most commonly uses a UV-Vis absorbance detector; however a wide range of other chromatography detectors can be used.

2.10 Analysis of variance (ANOVA)

ANOVA is a statistical technique used to analyses relationship and variation between quantitative dependent variable and qualitative independent variables. It was used to estimate the significance of model coefficients. Sir Ronald Fisher pioneered the development of ANOVA for analyzing experimental results (Fisher, 1974). The objective of ANOVA is to test whether the response means are identical across factor levels. A replication means that when two or more independent experimental units are utilized for a factor level (Larson, 2008).

From the statistical analysis using ANOVA, the program suggests the best fitted model and provides a response graph for the measured response. The quality of the polynomial regression model was expressed by the coefficient of determination (R2) and its adjusted value (Ad R2). The R2 coefficients value is 0 to 1, indicating the range of percentage of the variability in the response that could be explained by the model. The Fisher, F-ratio, is the ratio of the regression mean square to the mean square error. It is a variance ratio performed to test the significance of the regression model under

investigation with respect to the variance of all the terms included the error at the desired significance level (Montgomery, 2001).

The probability value, P-value determination is to test the risk of falsely rejecting a given hypothesis. A “Prob. > F” value on the F-test indicates the expected time proportion to get the stated F-value if no significant effects of factor. A statistical test can be performed to indicate the significance of the replicate error in comparison to the dependent error of the model as the replicate measurements are available (Montgomery, 2001). In this test, the residual or error of sum of squares is divided into two portions, first which is due to pure error based on replicate measurements and the second is due to lack-of-fit based on performance of model. The lack-of-fit test is a ratio of lack-of-fit mean square to the pure error mean square. An insignificant lack-of-fit is desired as it indicates there is no contribution in the regressor-response relationship accounted by the model (Noordin et al., 2004).

Additionally, the model adequacy is investigated by the residuals examination which shows the difference between the observed and predicted responses using the normal probability plots of the residuals and residuals versus the predicted response plot. A straight line is generated on the normal probability plots in an adequate model while the residuals versus predicted response plot should contain no obvious patterns (Montgomery, 2001).

2.11 Optimization Review

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 at al., 2008). In order to scale-up the biochemical xylitol purification, the process should be optimized. Commonly used optimization techniques are briefly described below:

2.11.1 One-Factor-At-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 constant value. This optimization technique is called one-factor-at-a-time (OFAT) (Bezerra et al., 2008). According to Montgomery (2001), OFAT method is 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, Zuriana *et al*. reported that OFAT is used to determine the possible optimum level of parameters for further optimization of sorbitol purification by central composite design (CCD) under response surface methodology (RSM). Literature survey revealed that sequential studies with OFAT method and RSM in purification of xylitol (Zuriana & A.Sidi, 2013) It is, therefore, justified to employ a sequential optimization studies involving OFAT approach and statistical design for the improvement of xylitol purification which is similar group of polyol with sorbitol in nature, via liquid-liquid extraction.

2.11.2 Response Surface Methodology

To detect the effects of interaction among the variables, the optimization of purification 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 technique 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 influences by several variables. The purpose is to simultaneously optimize the levels of these variables to achieve the best process performance (Bezerra et al., 2008).

2.11.3 Central Composite Design

According to Bezerra et al. (2008), CCD is the most employed design of optimization for the development of analytical procedures compared to the others as their low efficiency of the latter especially for a numbers of variables. CCD is a second order factorial design utilized in RSM since full factorial design (FFD) possessed too large number of runs which is less practical (Box & Wilson, 1951). The design composed of a full factorial, an additional design in which experimental points are located at a distance from center point and a center point. The experiment number is based on the
Number of parameters as expressed as number of parameters as expressed as

$$N = k^2 + 2k + Cp \qquad (2.3)$$

Where k is the parameter number and cp represents the replicate number of central point. The replication of central point is to estimate experimental error while axial points are to ensure the rotate ability of the design. All design factors are codified in 5-levels which are –α, -1, 0, +1, +α. In this study, the ind (2.3) where k is the parameter number and cp represents the replicate number of central point. The replication of central point is to estimate experimental error while axial points are to ensure the rotate ability of the design. All design factors are codified in 5-levels which are –α, -1, 0, +1, +α. In this study, the independent variables number are two, thus there are 13 experiments to be completed including five center points. The factorial design for this experiment is (-1), center point is (0, 0) and star point is (1) (Gunst, 1996). CCD for two variables and three variables optimization are shown in Figure 2.7 in (a) and (b), respectively.

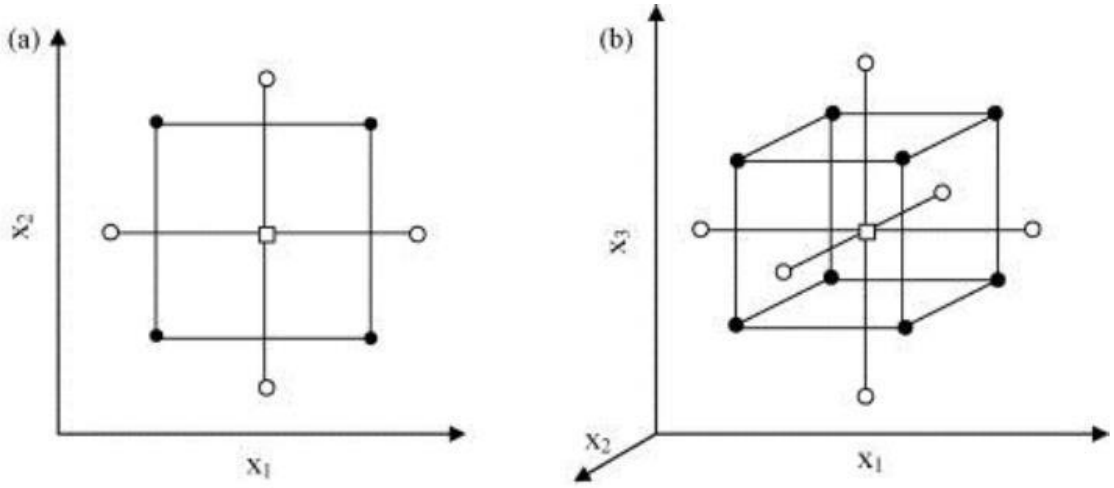
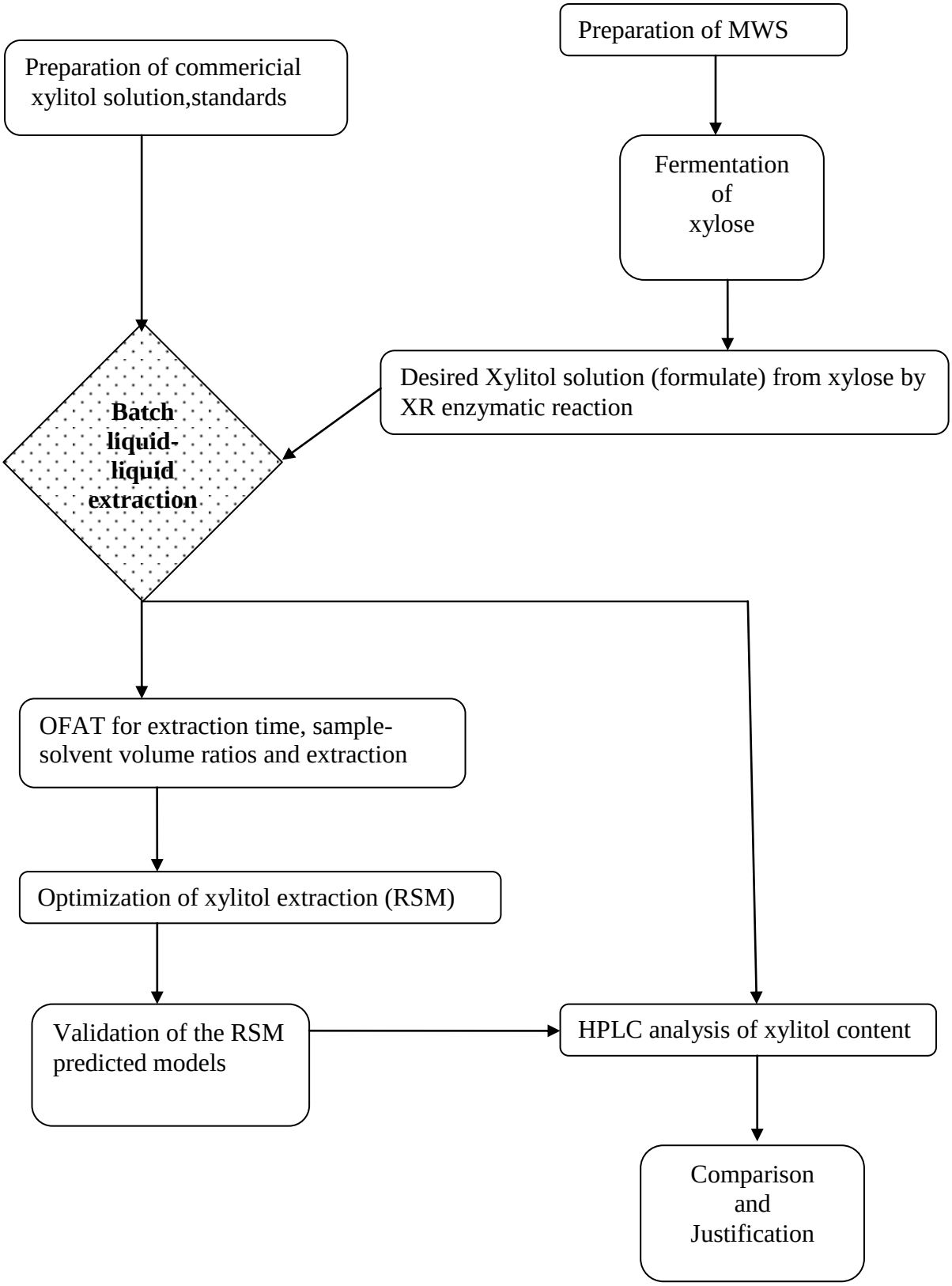
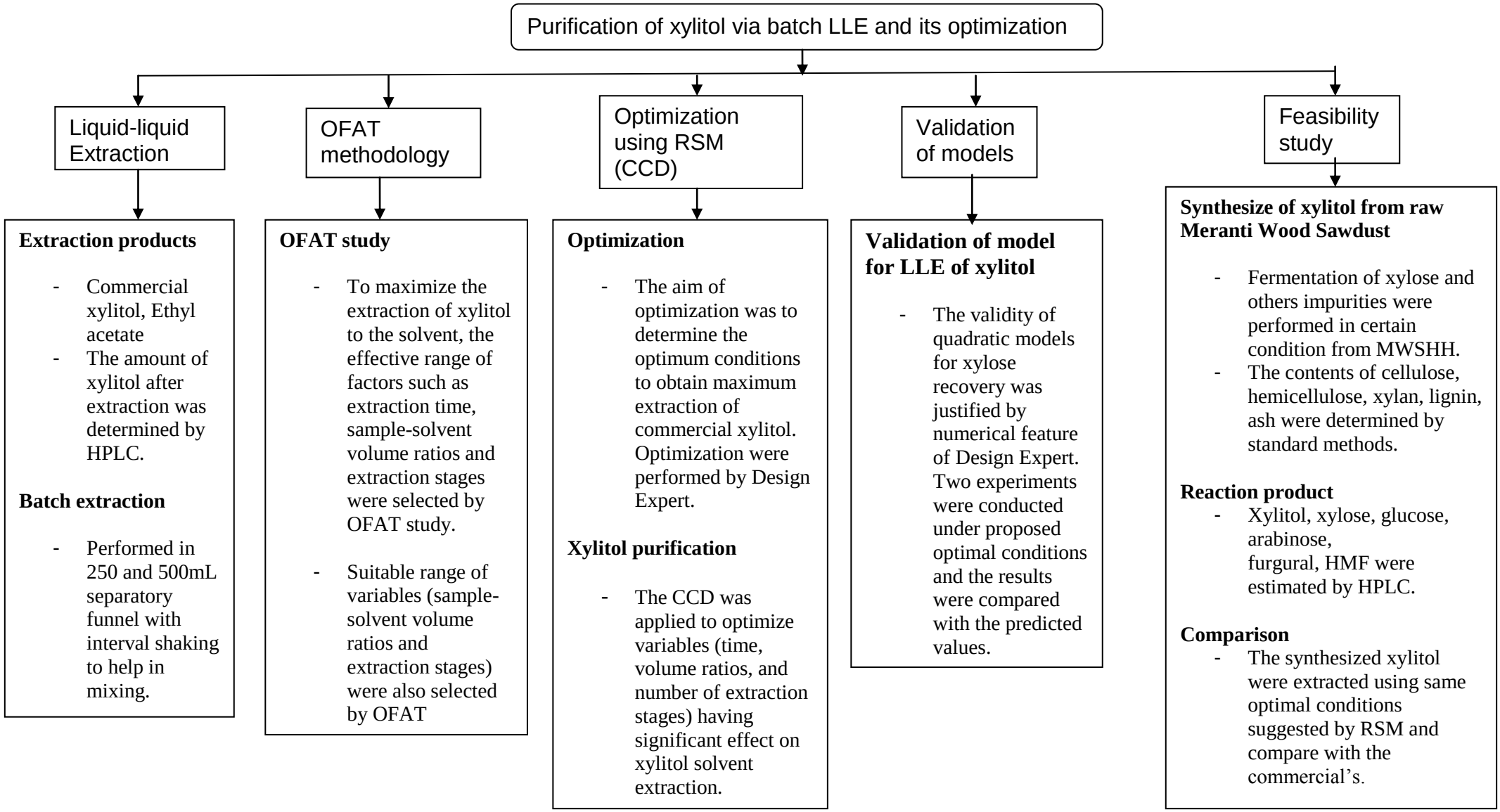


Figure 2.6 : Central composite designs for the optimization of: (a) two variables and (b) three variables. (●) Points of factorial design, (○) axial points and (□) central point

3.2 Experimental flowchart



3.3 Study operational framework diagram



3.4 Sample preparation

For commercial sample preparation, required mass of commercial xylitol was dissolved with calculated volume of distilled water to get desired 67 g/L concentration (Misra *et.al*, 2011) as stock solution. Different volume of stock solution was prepared accordingly based on OFAT and RSM experiments along the research.

For synthesize sample preparation, after pre-treatment of MWS with sulphuric acid, MWS hemi-cellulosic hydro lysate (MWSHH) was boiled at 100°C on a hot plate and its volume was reduced in the xylose content (37.6g/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 0.1M potassium phosphate buffer (pH7.0), crude XR enzyme, and NADPH in 50mL Erlenmeyer flask as reported by Yokoyama et al. (1995). The reaction was started by the addition of MWSHH as substrate. Pre-boiled XR was used instead of fresh XR as control. Following through mixing of the reaction mixture, a 10μL volume was withdrawn to use as a zero time reaction, boiled and then stored at -20°C 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°C for 5 min. The denatured protein in the reaction sample was separated by centrifugation at 8000rpm for 10min at 4°C. The supernatant was stored at -20°C and analysed for xylitol and impurities sugar content (Rafiqul, 2012).

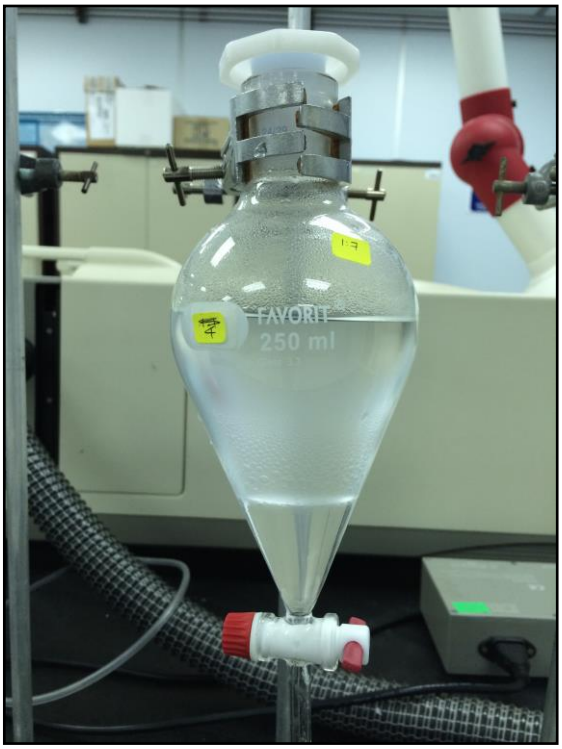


Figure 3.6: Extraction using separatory funnel

3.6 Standard Curve

Extracted xylitol solutions were diluted to 1:10 dilution factors with ultrapure water and then filtered with nylon syringe filter (0.22 μm Millipore Corp., Bedford, MA). Then preparation of different concentration of commercial xylitol solution ranging from 0.1M – 1.0M. The absorbance reading will be recorded based on the RI detector in the HPLC. A calibration curve is then develop as a standard. (Suzana, 2012)

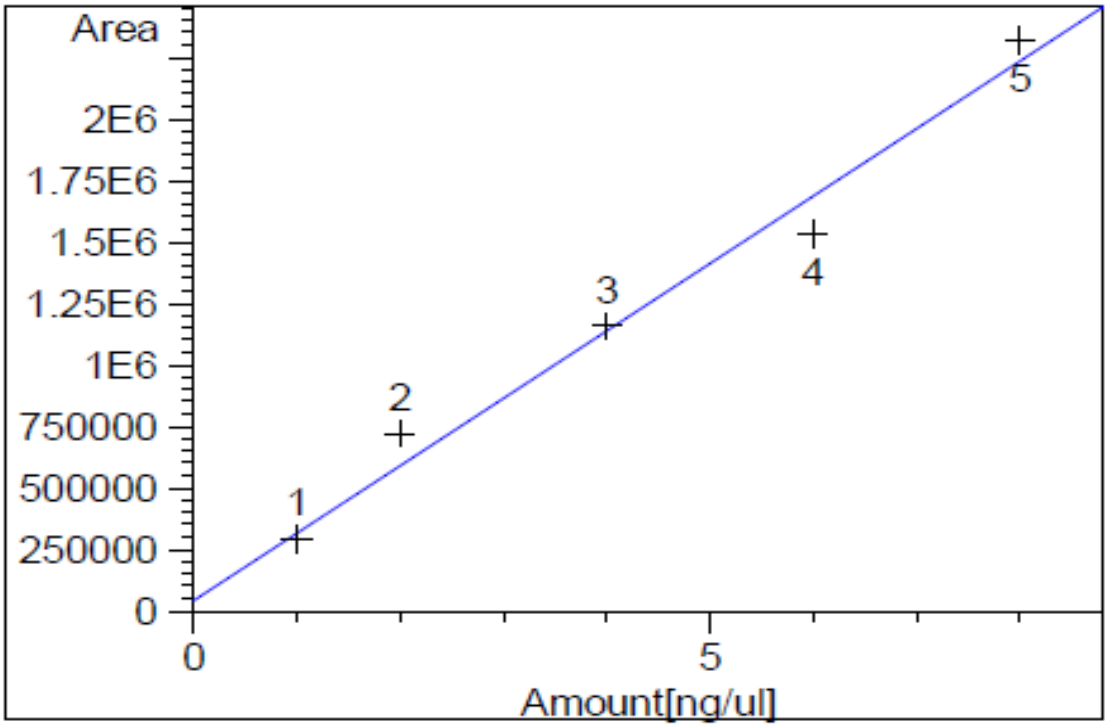


Figure 3.7: Calibration curve for xylitol

3.7 Experimental design (One-Factor-At-Time)

Assays of the commercial xylitol is to be carry out before starting of the experiment to identify the composition initially Extraction conditions for xylitol with ethyl acetate, namely volume ratio of xylitol to solvent, xylitol concentration, extraction time, will be determine by varying one factor at a time while keeping the others constant for delimitation of the experimental region. The starting fixed factors are the volume ratio of xylitol-to-solvent of 1:5 and xylitol solution concentration of 66.78g/L. (Table 3.1)

Table 3.1 : Literature data of xylitol to solvent volume ratios and initial xylitol concentration
(Misra *et al.*, 2011)

Organic solvent	Ratio (broth to organic solvent)	Xylitol concentration in aqueous phase (g/L)	Xylitol concentration in organic phase (g/L)
Ethyl acetate	1:1	48.07 ± 1.92	17.01 ±0.340
Ethyl acetate	1:3	46.52 ±1.86	18.91 ±0.567
Ethyl acetate	1:5	43.58 ±1.30	21.72 ±0.868
Ethyl acetate	1:7	43.74 ±1.31	21.98 ±0.879

Initial xylitol concentration : 66.78 g/L

Figure 3.8: OFAT experiment set up

3.7.1 Extraction time evaluation

Samples were extracted with ethyl acetate, following the procedures as described in Section 3.5. The independent variables were constant sample concentration (66.78g/L) (Misra *et al*., 2011), and sample-to-solvent ratio of (1:5) and extraction temperature (25°C). The optimal extraction time was selected from range 45, 60, 75, 90, and 105 min (Tan *et al*., 2013) upon the highest value of xylitol concentration at aqueous phase after extraction (g/L).

3.7.2 *Extraction sample to solvent volume ratio evaluation*

Extraction time of 60 min in Section 3.7.1 was selected for the following experiments. Using different sample-to-solvent volume ratios, ranging from 1:1, 1:3, 1:5, 1:7, and 1:11 by the same procedure described in Section 3.5 while constant out the other independent variable, which are the extraction stage of 1 stage. The optimal sample-to-solvent volume ratio was selected upon the lowest value of xylitol concentration at aqueous phase after extraction (g/L).

3.7.3 Extraction Number of stages evaluation

Based on the sample to solvent volume ratio and initial xylitol concentration selections in Sections 3.7.1 and Section 3.7.2 and by repeating the same procedure as described in Section 3.5, the extraction process will be carried out at different extraction stages: 1, 2, 3,4,5,6,7,8,9 and 10 stages. The optimal extraction staging was selected upon the first constant lowest value of xylitol concentration at aqueous phase after extraction (g/L). After 3 settings of employing one variable at a time method, 3 optimum values will be obtained and each of these values will be set as midpoint by giving upper and lower boundaries. These 3 optimized ranges will be use in the response surface methodology analysis later and the first factor which is the extraction time proven to be insignificant to the response and thus screened out.

Table 2 represents the experimental design of process factors in the fashion of one-factor-at-time. OFAT is important in determining the midpoints for each factor to be used in RSM analysis later, it also serves to screen out the process factor that is not or have negligible effects on the research response. The extraction time is shown to be insignificant to the extraction of sample with solvent after OFAT being carried out. Table 2 indicated that for OFAT design the ranges used for extraction time from 45-105 min with 15 min intervals, meanwhile for volume ratios of sample to solvent ranged from 1:1 to 1:11 in which 1:1, 1:3, 1:5, 1:7, 1:11 were selected. Lastly, extraction staging was ranging from n=1 to n=9 with one stage intervals. The experiments were carried out in sequential with extraction time then volumes ratios then number of stages and were all run in triplicate. Three settings of OFAT generated 3 optimum values and each of these values will be set as midpoint by giving upper and lower boundaries in RSM analysis later.

Table 3.2 : Design of process variables and their ranges used in OFAT study for xylitol purification

	Variation of variables		
Factors	Extraction time (min)	Volume ratios of sample solution to ethyl acetate	Extraction : number of stages (N)
Extraction time	45-105	1:5	1
Volume ratios: sample solution	xx	1:1 – 1:11	1
to EtAC	xx	yy	1 – 9
Extraction : number of stages			
OFAT midpoint	xx	yy	zz

Note: xx, yy and zz are possible value of extraction time (factor 1), volume ratios of sample solution to ethyl acetate (factor 2), and number of stages (factor 3) respectively.

3.8 Design of Experiment (RSM)

The two factors of the sample to solvent volume ratios (A) and number of extraction stages (B) were used to optimize the purification of xylitol. RSM using Design Expert V7.0 was introduced for analysis of optimization in the experiment. Under RSM, CCD was selected to insert response results. These two independent variables involved in CCD with preset range and levels are shown in Table 3.2. Next, an experimental design table was constructed. Then, experimental run was sorted in standard order to avoid bias as illustrated in **Table 3.2**. All the experiments were run in triplicate. Data obtained from lab experiment were inserted into the response column and were analyzed statistically using ANOVA. After the suggested optimum conditions has obtained, validation run was conducted.

Table 3.3 : Independent variables involved in Central Composite Design (CCD).

Independent variable	Range and level				
	2.00(-α)	**-1**	**0**	**+1**	**2.00(α)**
sample to solvent volume ratios	1:2	1:3	1:4	1:5	1:6
number of extraction stages	2	3	4	5	6

3.8.1 RSM analysis: experimental set up

Response surface methodology (RSM) is a summation of experimental strategies, mathematical methods and statistical inference widely used for optimization steps in biochemical processes (Chen, He, & Ali, 2002). RSM evaluate the mutual effects of several affecting factors at different levels and determining a wide region in which the obtained results are valid (Montgomery, 2006). ANOVA statistical test will be carried out using Design Expert software version 7.0 to determine the optimum conditions for maximum xylitol extracted with ethyl acetate. From the RSM analysis, new optimum conditions are obtained and experiment was conducted based on the analysis result. 13 sets of experiment were run at different weeks. Five runs and four runs and four runs were conducted total up 13 experiments with triplicates running for each set of experiments.

Table 3.4 : Optimization design of CCD in Design Expert V7.0 software.

Run	Standard	Factor 1: Xyl:EtAc ratio (v/v)	Factor 2: No. of Extraction stages (n)
1	7	1:2	4
2	1	1:4	4
3	4	1:4	4
4	11	1:3	5
5	12	1:4	6
6	2	1:4	4
7	13	1:4	4
8	10	1:6	4
9	9	1:5	5
10	6	1:4	2
11	3	1:3	3
12	8	1:5	3
13	5	1:4	4

Optimization of factor 1 and factor 2 were completed using RSM in Design Expert Software and the results were presented at Chapter 4.

Figure 3.9 : RSM experiment set up

3.8.2 Validation experimental set up

The optimum condition for xylitol content after extraction depended on the sample to solvent volume ratios and the number of extraction stages were obtained from the predictive equations of CCD. By using the optimized parameter set points, the experiment was carried out once again. The optimum condition predicted by CCD in Design Expert V7.0 was volume ratios of 1:5 and 5 stages of extraction for the xylitol extraction process. The experimental and predicted values were compared in Table 3.4 in order to determine the validity of the model.

Table 3.5 : Validation experiment condition

Factor 1: **Volume ratios**	**Factor 2:** **Number of stages**
1:5 **1:5**	**5** **5**

3.9 Analytical Method

Extracted xylitol solutions were diluted to 1:10 dilution factors with ultrapure water and then filtered with nylon syringe filter (0.22 μm Millipore Corp., Bedford, MA). Xylitol concentration after extraction and impurities were separated by high performance liquid chromatography (HPLC). The analysis was carried out using an Agilent 1200 chromatograph (Agilent, USA) equipped with a refractive index detector (RID). A RezexRHM Monosaccharide H+ column (50mm × 7.8mm; Phenomenex, USA) operated at 80 °C. Ultrapure water was filtered and undergoes ultra-sonication to be used as mobile phase at a flow rate of 0.6 mL/min and 20 μL of diluted sample was injected by auto sampler. The peak areas were determined by absorbance at a specific wavelength. Xylitol concentration was calculated as % area relative to total area (nRIU*s).

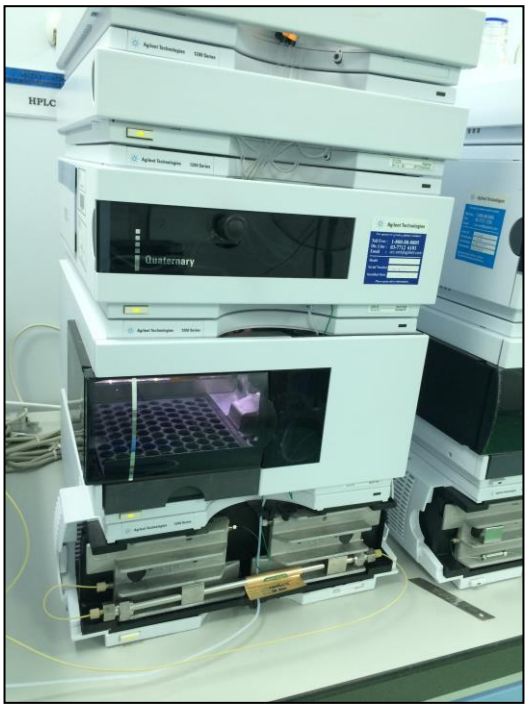


Figure 3.10 : HPLC Agilent 1200

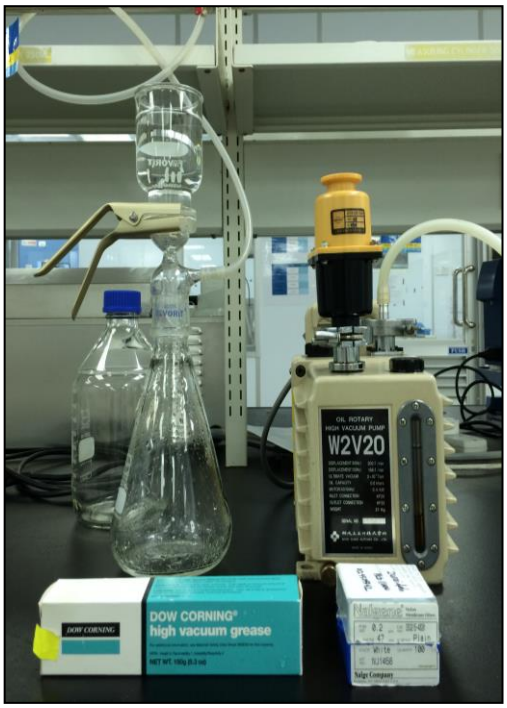


Figure 3.11 : Mobile Phase preparation (Vacuum filtration)

3.10 Xylose fermentation: feasibility study

3.10.1 Acid hydrolysis of MWS

3g of MWS on an oven dry basis (o.d.b.) was mixed with the required amount of sulphuric acid solution (%, w/w) in 250mL 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. 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 has elapsed, the autoclave was cooled down to 95 °C.

Figure 3.12: *Autoclaving MWS broth*

3.10.2 Enzymatic xylitol synthesis method

MWS hemicellulosichydrolysate (MWSHH) was boiled at 100degC on a hot plate and its volume was reduced in the xylose content (37.6g/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 0.1M potassium phosphate buffer (pH7.0), crude XR enzyme, and NADPH in 50mL 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 through mixing of the reaction mixture, a 100miuL volume was withdrawn to use as a zero time reaction, boiled and then stored at -20degC 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 100degC for 5 min. The denatured protein in the reaction sample was separated by centrifugation at 8000rpm for 10min at 4degC. The supernatant was stored ay -20degC and analysed 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.

Figure 3.13: Fermentor used : 200mL

4 RESULTS AND DISCUSSIONS

4.1 HPLC result and discussion

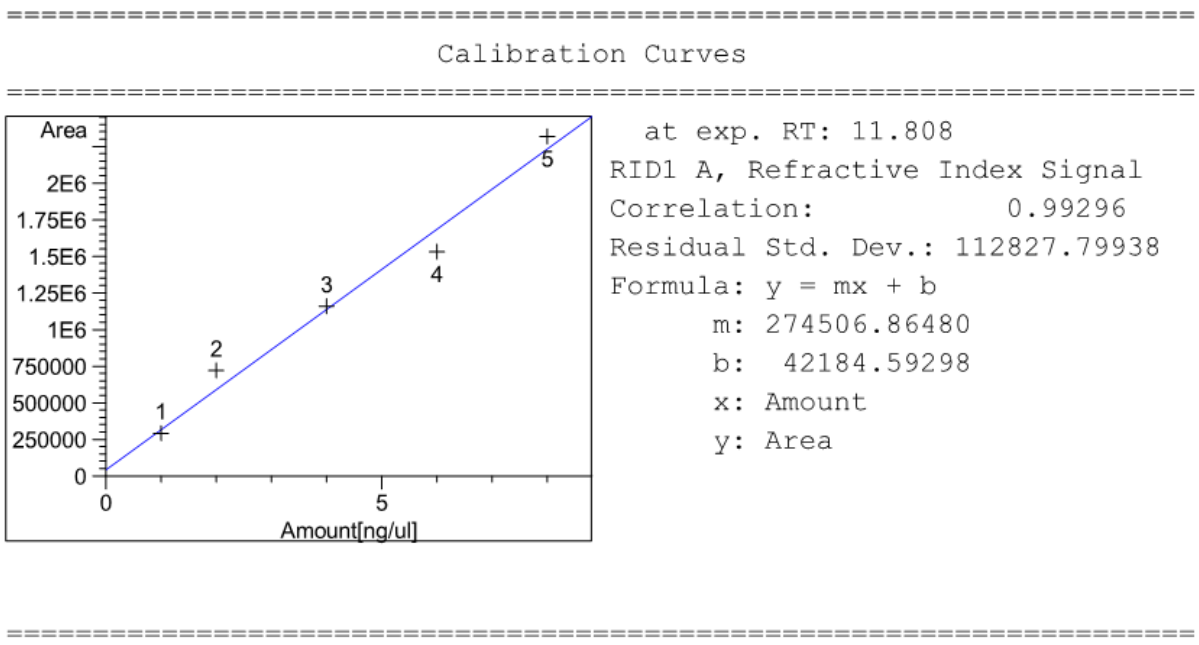


Figure 4.1: Calibration curve for xylitol

Extracted xylitol solutions were diluted to 1:10 dilution factors with ultrapure water and then filtered with nylon syringe filter (0.22 µm Millipore Corp., Bedford, MA). Xylitol concentration after extraction and impurities were separated by high performance liquid chromatography (HPLC). The analysis was carried out using an Agilent 1200 chromatograph (Agilent, USA) equipped with a refractive index detector (RID). A Rezex RHM Monosaccharide H+ column (50mm × 7.8mm; Phenomenex, USA) operated at 80 °C. Ultrapure water was filtered and undergoes ultra sonication to be used as mobile phase at a flow rate of 0.6 mL/min and 20 µL of diluted sample was injected by auto sampler. The peak areas were determined by absorbance at a specific wavelength. Xylitol concentration was calculated as % area relative to total area(nRIU*s). The xylitol concentrations were identified by mean of retention time and peak were quantified by comparing peak areas with the results of calibration series using pure xylitol standards obtained from Sigma-Aldrich. Xylitol calibration standard

was prepared by serial dilution using ultra-pure water. There was a linear response between xylitol concentration and RI absorbance at for all standards over the calibration range studied as shown in Figure 1. For the purpose of quantitation,standard curves were constructed using a line fit forced through zero and the correlation coefficient was r^2= 0.99 for xylitol.

Figure 4.2 illustrate the HPLC chromatogram for different experiment conditions of xylitol extraction being conducted

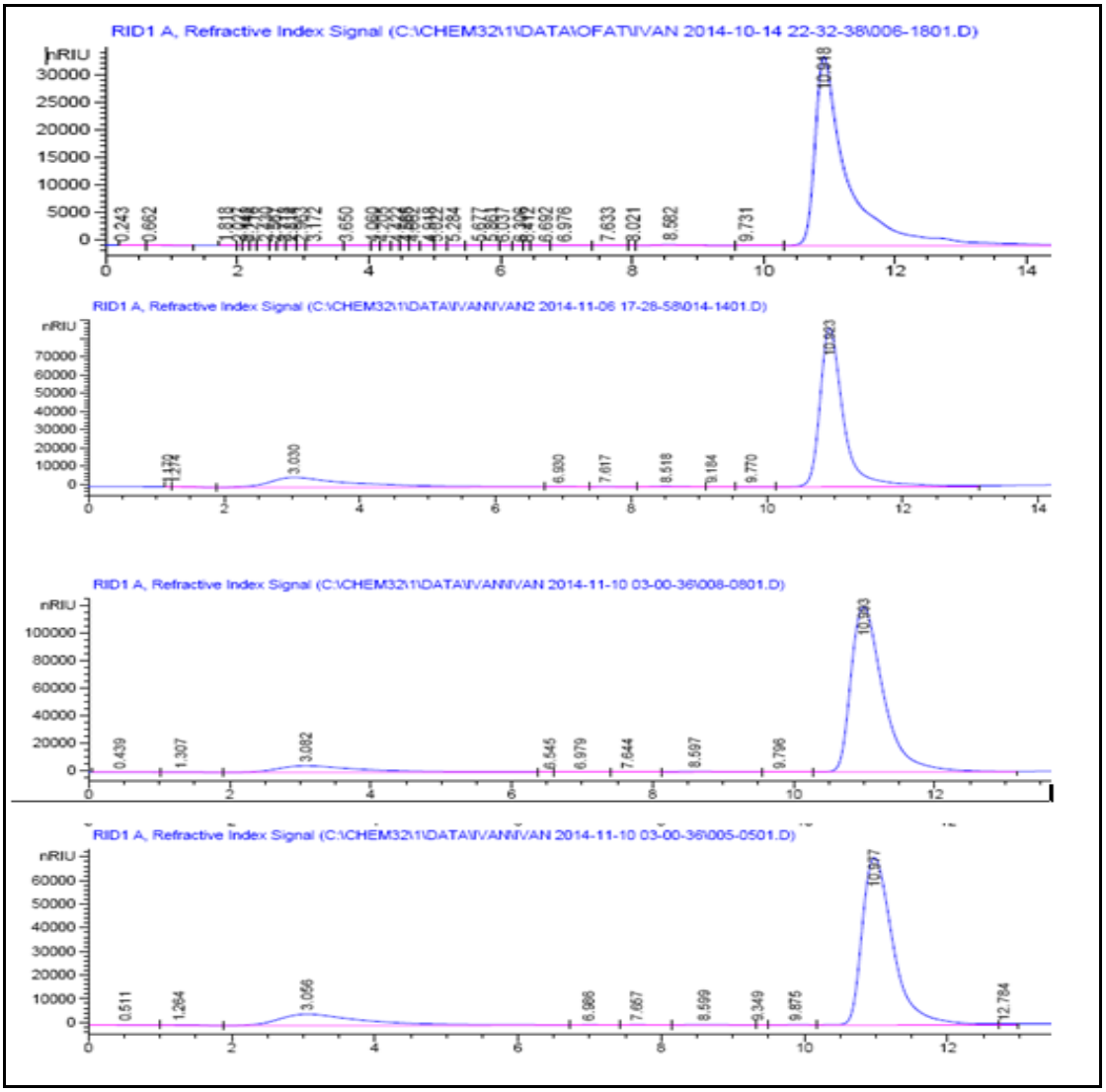


Figure 4.2: HPLC chromatogram of standard solution (a), extracted xylitol without optimization(v/v=1:1, n=1) (b), extracted xylitol with optimization (c), and synthetized xylitol content (d).

Signal 2: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Area %
1	0.252	BV	0.1362	736.33575	0.0318
2	0.436	VV	0.8378	8023.19385	0.3470
3	1.242	VV	0.0872	1648.35217	0.0713
4	1.314	VV	0.4204	6509.29541	0.2815
5	3.077	VV	1.2524	3.36679e5	14.5608
6	6.934	VV	0.4894	1.02765e4	0.4444
7	7.621	VV	0.4856	9419.50684	0.4074
8	8.521	VV	0.6172	1.52467e4	0.6594
9	9.796	VV	0.6887	1.09506e4	0.4736
10	10.923	VV	0.3630	1.91275e6	82.7228
11	11.808		0.0000	0.00000	0.0000
Totals :				2.31224e6	

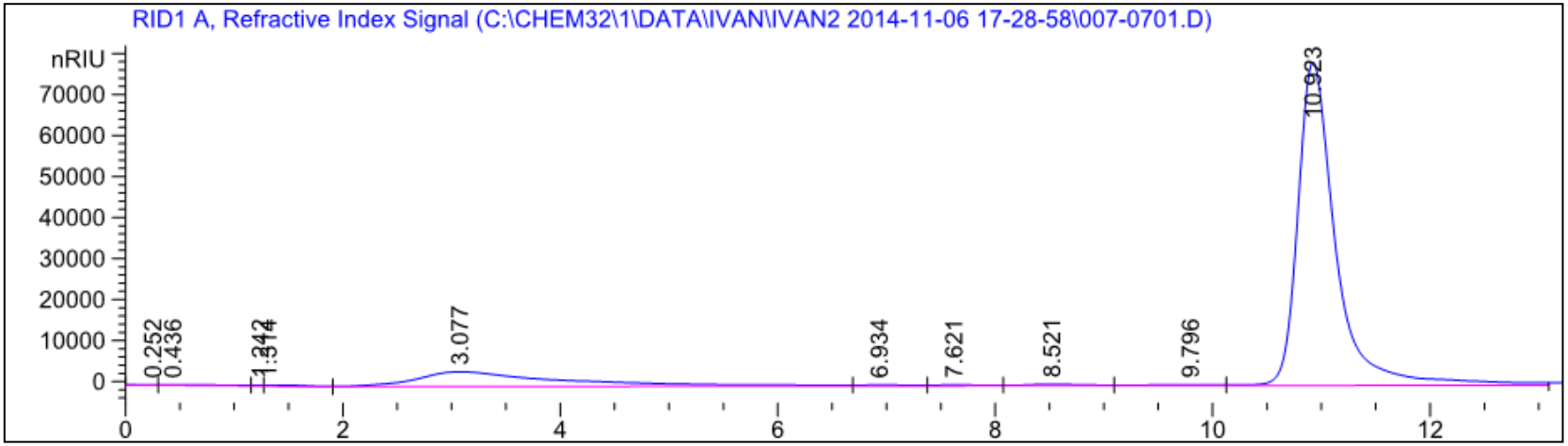


Figure 4.3: HPLC chromatogram (a), and Area (nRIU*s) (b) for random picked sample of volume ratio 1:3

4.2 OFAT result and discussion

Comparison of HPLC chromatogram was made between the standard and sample of xylitolto quantify xylitol composition in each sample. The major peaks, corresponding to xylitol appeared at elution times within 10 min and 11 min (see Fig. 2). The percentage of xylitol extracted is calculated as in equation 1. Final mass is obtained by multiplying the final volume with the concentration of xylitol of the sample in the aqueous layer analyzed by HPLC. The raw data representation refer to appendix A-2.

$$\text{Xylitol \% extraction} = \frac{Initial\ mass\,(g) - final\ mass\,(g)}{Initial\ mass\,(g)} \times 100 \qquad \text{(eq.4.4)}$$

Final mass(g) = final volume in aqueous (mL) × concentration from HPLC (g/L) (eq.4.5)

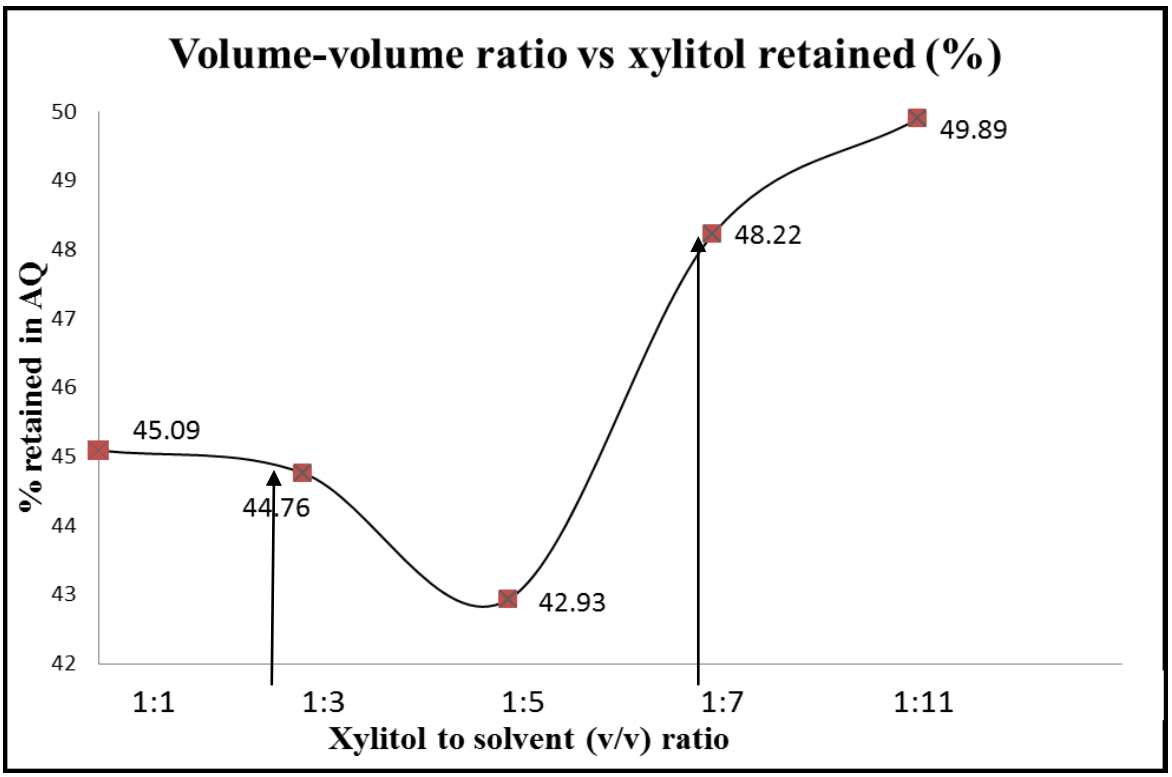


Figure 4.4: Xylitol remaining % in aqueous layer after extraction vs. sample-solvent volume ratios

To note, first factor which is the extraction time was screened out after experiments conducted at one-factor-of-time due to the insignificant results obtained for different extraction time varying from 45 min to 105 min. Raw data presented at Appendix A. Figure 3 shows the percentage of xylitol remaining in aqueous layer after extraction with varying different sample to solvent volume ratios. For sample to solvent volume ratios of 1:1 and 1:3, the removal of xylitol is recorded at 55 and 56 % respectively. This suggested that the greater proportion of solvent used, the greater the extraction can be. The results suggest that a volume ratio of 1:5 in total volume of 240mL yielded the highest extraction of xylitol to the organic layer with 58% removal.The result obtained is in the same trend as research from Misra and coworkers with extraction peak at 1:5 volume ratios.

Further increasing the solvent volume resulted in lesser extraction of xylitol to the ethyl acetate site as shown in Figure 3 i.e. merely 50% removal and at volume ratios of 1:11 insignificant increment of removal of xylitol observed. This may attributed by the greater liquid-liquid film resistance formed accompanied by the increased in emulsion formed as the volume of solvent is increased which contributed greater barrier for the xylitol to be extracted effectively (Mussato *et. al*., 2006). Similar trends were observed for triplicate trials. Therefore, volume ratio of xylitol to ethyl acetate of 1:5 was selected as the midpoint in the central composite design in RSM optimization and volume ratios of 1:2 and 1:6 were selected as the lower and upper range.

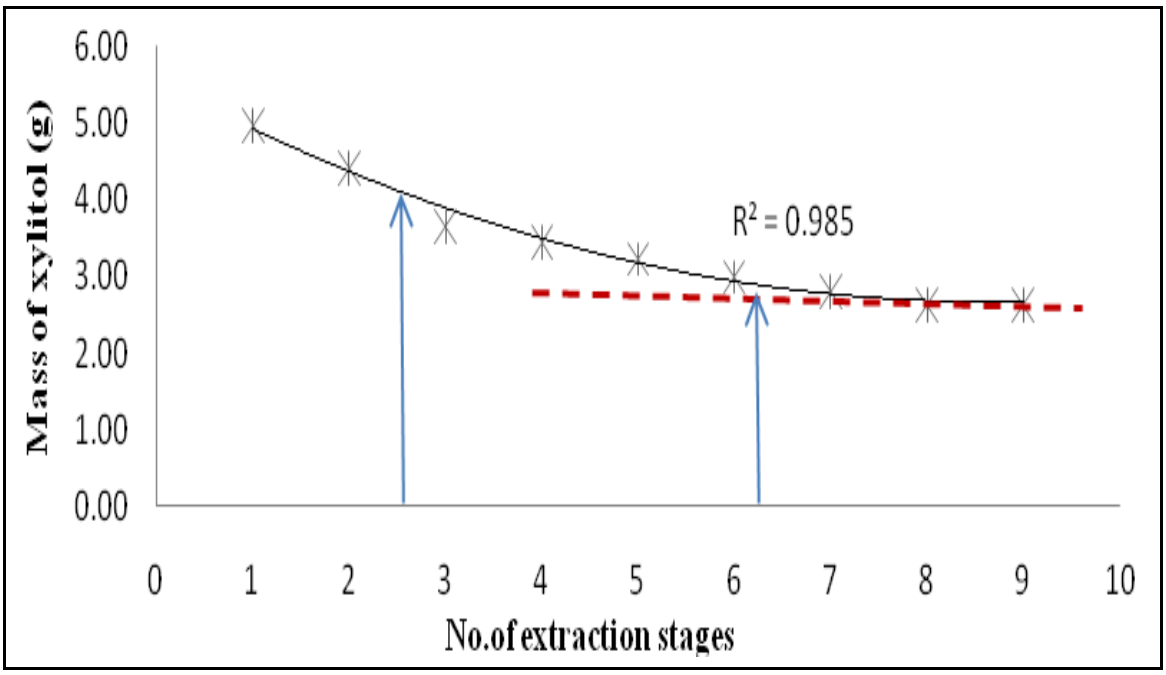


Figure 4.5 : Xylitol mass remaining in aqueous layer after extraction vs. number of extracting stages

Figure 4 shows the mass of xylitol remaining in aqueous layer after extraction at different number of extraction stages with volume ratio of 1:5 from previous OFAT result. The initial mass of xylitol in the aqueous layer before extraction was 5.28 g. From the graph, the mass of xylitol remained at the aqueous layer decreases along with the increment of the staging. This indicated that some xylitol was extracted to the fresh solvent added in each stage..

When two stages of extraction were carried out with addition of fresh solvent, the removal of xylitol increased to 16.7% to the ethyl acetate. The trend continued until 6 stages and became constant when 50.2% of xylitol successfully removed. This suggested that the maximum recovery of xylitol using ethyl acetate as solvent is around 50%. Multiple stages yielded higher extraction efficiency as it increases the extraction partition coefficient by decreasing the concentration of solute in aqueous layer by batch addition of fresh ethyl acetate referring eq.4.6.

$$K=\frac{[\mathrm{HA}_{organic}]2}{[\mathrm{HA}_{aqueous}]} \qquad \text{(eq. 4.6)}$$

The greater the extractability when the concentration of solute (xylitol) is lesser in aqueous layer. Therefore, 4 stages (n=4) of extraction was chosen as the midpoint in CCD for RSM analysis to optimize this factor to the purification of xylitol. 2 stages (n=2) and 6 stages (n=6) were selected as upper and lower range, note that the upper range can be any point after 6 stages as the response became constant but to save time, 6 stages was selected. Experiments were conducted in triplicate and raw data represented at Appendix B-2.

From OFAT result as in figure 4.4 and 4.5 , the significance ranges that shown effects to the response which were factor A: volume ratio of sample to solvent, factor B: number of extraction stages were selected and used as the maximum and minimum boundary in RSM Central composite design.

4.3 RSM model statistical analysis result and discussion

Each test with a new batch of xylitol sample was repeated in triplicate. Analysis of variance (ANOVA) and optimization correlation for two factors were performed by using the Design Expert software, and a least significant difference test was used to compare the means with a confidence interval of 95%.

4.3.1 Optimization studies with CCD

In this design of experiment, CCD was implemented for the optimization of biogas production. The two factors involved in this study were agitation speed and reaction time. By using CCD, a total of 13 runs were generated with different set up condition. The response of biogas yield attained from the experiment was tabulated in **Table 4.2**. These results data were input into the Design Expert V7.0 software for further analysis. By employing multiple regression analysis on the experimental data, the optimization

result data generated from RSM was fitted with a second order polynomial equation as shown in equation (4.7).

Remaining xylitol mass (g) = -0.13039 + 2.12578 × 10-3 A + 3.75473 × 10-3 B – 1.38744 × 10-6 AB – 8.81116 × 10-6 A2 - 5.42927 × 10-4 B2 (eq. 4.7)

Where A and B represent volume ratios and number of extraction stages respectively.

Table 4.3 : Result of optimization of xylitol extraction process in CCD.

Run	**Factor 1: Xyl:EtAc ratio (v/v)**	**Factor 2: No. of Extraction stages (n)**	**Remaining xylitol mass (g)**
1	1:2	4	2.471
2	1:4	4	2.257
3	1:4	4	2.262
4	1:3	5	2.292
5	1:4	6	2.315
6	1:4	4	2.253
7	1:4	4	2.265
8	1:6	4	2.189
9	1:5	5	2.223
10	1:4	2	2.355
11	1:3	3	2.361
12	1:5	3	2.312
13	1:4	4	2.258

4.3.2 Statistical Analysis

In order to analyze the results obtained, there are three tests need to be performed, which are test for significance of the regression model, test for significance on individual model coefficients and test for lack-of-fit.

From ANOVA result summarized in Table 4.3, the Model F-value of 11.31 and the *p*-value of 0.0030 implying the significant of model. There is only a 0.3% probability that a Model F-value this large could occur due to noise. This is desirable as it indicates the significant effect on the response of the model. In the same manner, both of the second-order effects which are volume ratios (A^2) and extraction stages (B^2) categorized as significant model terms with *p*-value less than 0.05. Other insignificant model terms can be terminated to generate an improved model. However, those models required to support the hierarchy are not counting.

The Sum of Squares for the Model source was 0.056, which represented the summation of Regression Sum of Squares for the quadratic regression model. Each regression source has corresponding degrees of freedom (DF) of one and hence contributes a total DF of 5 for the model source. The Mean Squares of the Model was 0.011, which was the division of Sum of Squares by the corresponding DF.

The Lack of Fit, F-value of 105.54 indicates the significant relative to the pure error. There was only a 0.03 % chance that it could occur due to noise. This means that there was some significant effect that has been neglected and that effect was a function of the factors which already existed in the model. A little change in the parameters might affect the fit of model. It was advisable to add more factors such as temperature and mixing efficiency in order to make the lack of fit to become desirably insignificant. Apart from that, it was recommended to widen the range of the parameters so that outliers can be included.

This model having a satisfactory R-Squared value of 0.8898 which implies the model was adequate for the design space navigation. The adequate precision measures the signal to noise ratio which compares the predicted values range at points of design to the average prediction error. A ratio greater than 4 is desirable for an adequate model. In this particular case, the ratio of 7.327 indicates adequate signal discrimination.

Table 4.4: Result for ANOVA.

Source	Sum of Squares	DF	Mean Square	F-value	*p-value*
Model	0.056	5	0.011	11.31	0.0030
A	0.039	1	0.039	39.36	0.0004
B	4.720×10^{-3}	1	4.720×10^{-3}	4.79	0.0647
AB	1.000×10^{-4}	1	1.000×10^{-4}	0.10	0.7593
$\mathbf{A^2}$	7.237×10^{-3}	1	7.237×10^{-3}	7.35	0.0302
$\mathbf{B^2}$	8.291×10^{-3}	1	8.291×10^{-3}	8.42	0.0229
Lack of fit	6.808×10^{-5}	3	2.150×10^{-5}	105.54	0.0003

4.3.3 *Residuals Analysis and Diagnostic Plots*

Residual analysis is necessary to ensure that the assumptions for the ANOVA are met. From the least squares fit, the residuals (*ei*) play a crucial role in judging the adequacy of the model and are defined by equation (4.8). The difference between the actual individual values is indicated as *yi* while the predicted value from the model is indicated as $\hat{y}i$.

$ei = yi - \hat{y}i$ where $i = 1, 2, 3, \ldots, n$ (eq. 4.8)

Diagnostic plots generated from CCD using Design Expert V7.0 are reviewed in residuals analysis to determine the feasibility of the model. The normality assumption may be checked by a normal probability plot of the residuals. The experimenter handbook by Kraber *et al.* (2002) stated that a good normal probability plot should shows a linear straight line whereas an S shape indicating a bad normal plot. The handbook also mentioned that good residuals versus predicted response plot should be random scatter whereas a bad plot of the kind will shows a megaphone shape. If the variance of the response depends on the mean level of *y,* then this plot will often exhibit

a funnel-shaped pattern. This is also suggestive of the need for transformation of the response variable *y*. A review on the normal probability plot for xylitol content after extraction as illustrated in **Figure 4.6** revealed that the residuals generally fall on a straight line implying that the errors are distributed normally. On the other hand, the residuals versus predicted response as shown in **Figure 4.7** revealed that they are random scattered without obvious pattern and unusual structure. This general impression implies that the model proposed was adequate and there was no reason to suspect any violation of the independence or constant variance assumption.

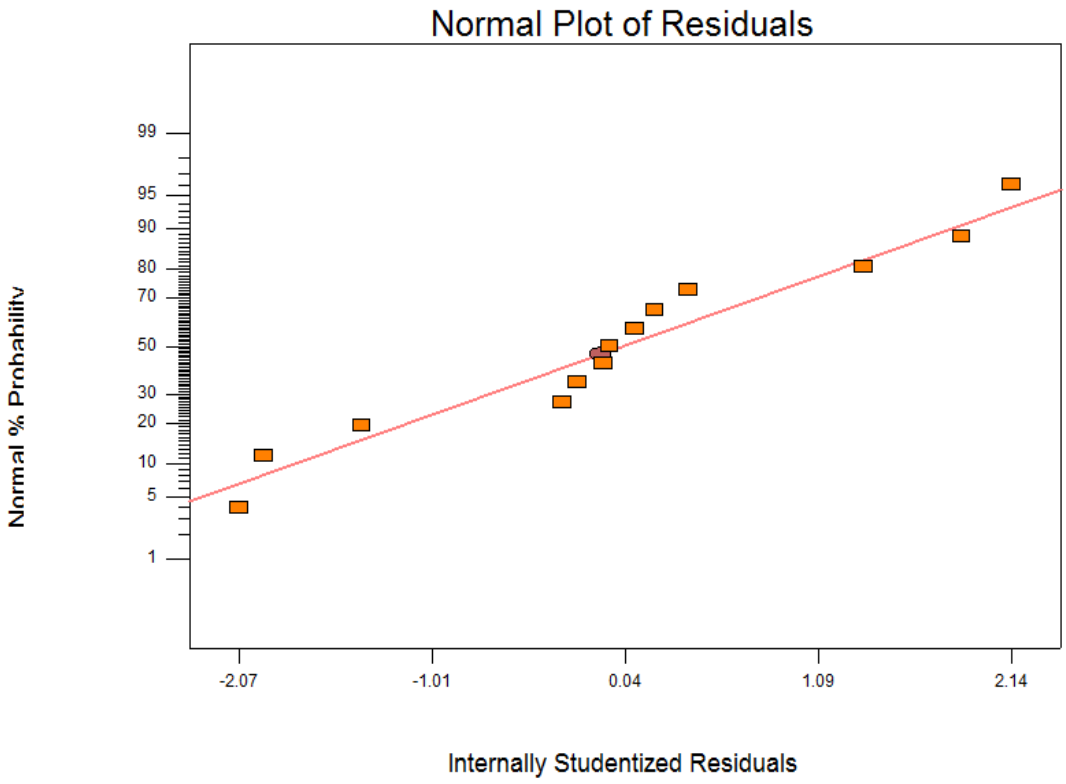


Figure 4.6: Normal probability plot of residuals for xylitol mass after extraction data.

extraction stages were greater than the centre point value, the reverse trend was observed.

The three-dimensional response surface graph generated in a perfect dome shape in which minimum points yield 2.212 g xylitol in aqueous layer as shown in Figure 4.4. This indicated the result obtained is optimized. This result in optimal conditions at xylitol to ethyl acetate volume ratios of 1:5 and extraction stages of 5 stages. Therefore, the optimization result data was used for this research study to be further validated.
In order to get a better understanding of the results, the response function for RSM data was assessed graphically by the use of perturbation plot. The perturbation plot helps to compare the effect of all the factors at a particular point in the RSM design space. It displays the effect of changing one factor from the reference point while holding the other factor constant. As can be seen from Figure 4.9, both volume ratios (A) and extraction stages (B) affected the remaining xylitol content in an almost similar trend of curvature. This indicates that both volume ratios and extraction stages factors showed significant quadratic effects that contributed to the xylitol solvent extraction.

For factor A, the remaining xylitol mass decreased up to a certain point, which is at coded unit of 0.000, and increased when the volume ratios increasing. Tailing of xylitol remaining mass trough surges due to greater volume ratio than the 0 coded units which might cause retardation in xylitol extraction from aqueous to organic layer. In this study, the effect of volume ratio to the optimization of xylitol extraction was crucial because volume ratios provides the essential different proportion of contact area for both layer in order for the solute i.e. xylitol to move from water to ethyl acetate. Mass and heat transfer also can be fostered by this volume ratio which can improve efficiency of mixing and in the other hand can degrade the mixing efficiency due to the formation of emulsion between layers with the increment of solvent being used in a system Besides In the present work, the solvents used for liquid–liquid extraction presented a tendency to form emulsion, turning the technique slow. (Mussatto et al, 2006). Liquid-liquid film resistances played the major role in aspect of mass transfer of xylitol from layer to layer (Geankoplis, 2003), meanwhile for the heat transfer view of aspect, the effect is not investigated in this research study however the temperature of extraction process can be included for future study in order to increase the lack of fit test of the model until a desirable level.

On the other hand, for factor B, the remaining xylitol mass showed an downward trend when the extraction staging increased. However, the tailing of falling trend started to slow down after the coded unit range of 0.000 to 0.500. Extraction stages can be considered as another vital factor in the determination of optimum condition for xylitol extraction process. This is due to the fact that freshly added solvent served to extract the leftover xylitol in the aqueous layer by the previous stage of extraction using the same solvent, the partition coefficient reached a saturation value when only one stage of extraction was performed. The concentration of solute in organic layer served as the 'driving force' in extracting the solute in an significant amount, the lesser the concentration in organic layer, the greater the extraction of solute to it. (Geankoplis, 2003)

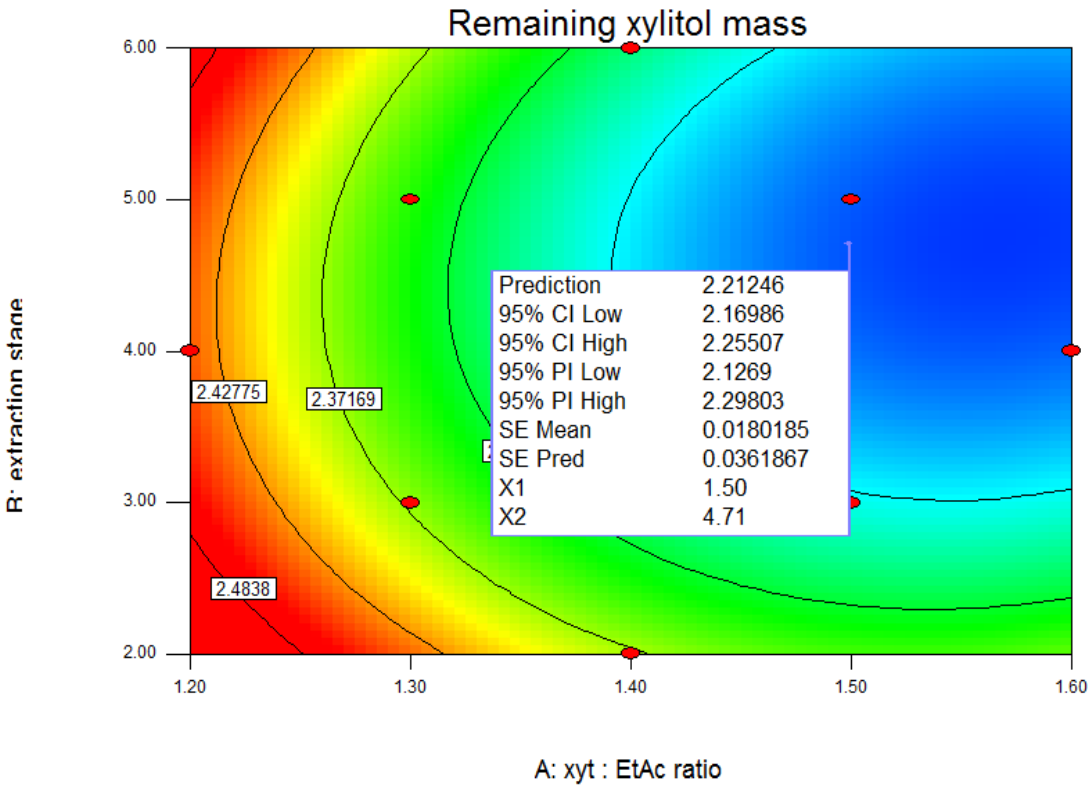


Figure 4.8: Contour plot graph of optimization.

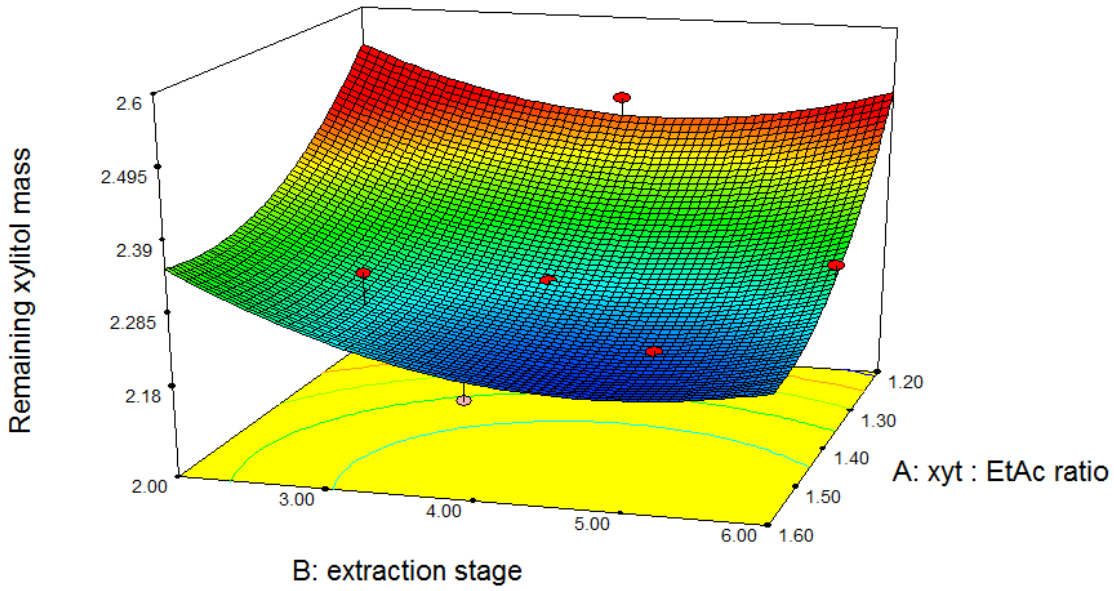


Figure 4.9: Three dimension model graph of optimization.

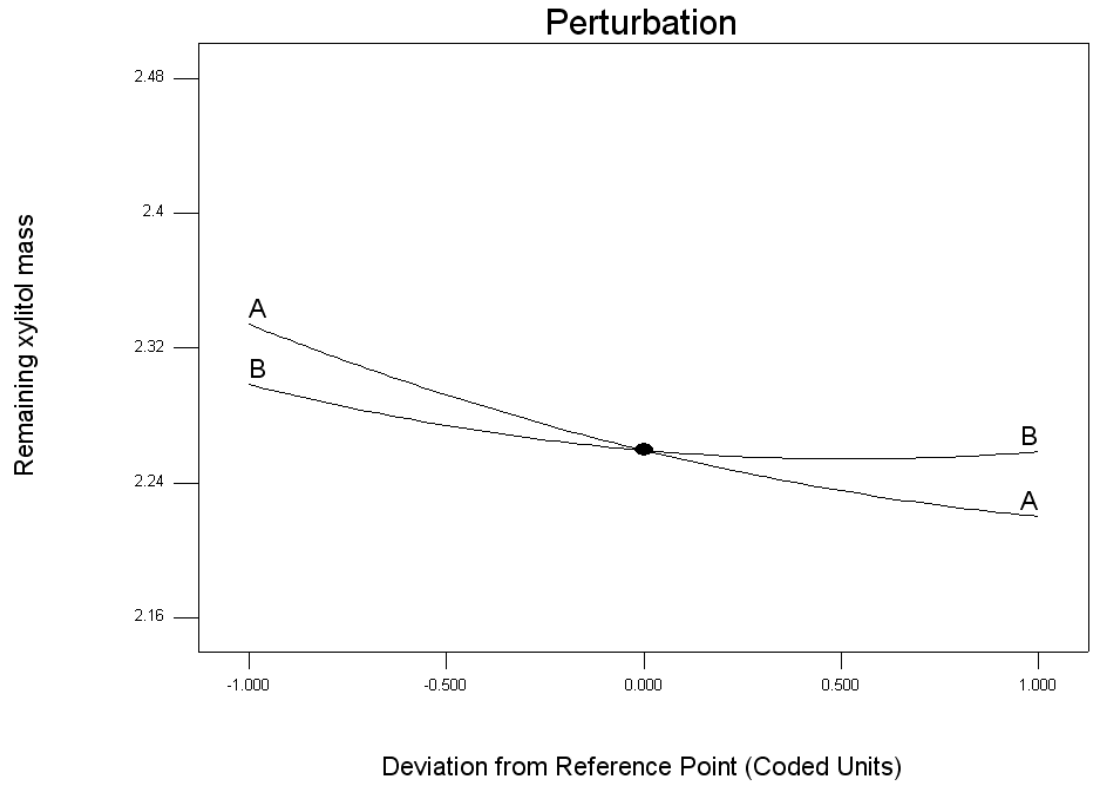


Figure 4.10: RSM Perturbation plot for remaining xylitol mass.

4.5 *Interaction of factors*

The interaction effect of xylitol to Ethyl acetate ratio and extraction stage to remaining xylitol mass.is plotted as in Figure 4.6. The non-parallel lines displayed in the interaction plot indicated that there was an interaction effect between volume ratios (A) and extraction stage (B) on xylitol extraction. According to Bakeman (2005), the less parallel the lines are, the most likely there is to be a significant interaction. In Figure 4.6, the lines are not parallel and there is no cross-over interaction, but an interaction would be expected when value of A approaching 1:1. The response grows curvilinear when the volume ratio increasing at a fixed level of extraction stage factor. At lower coded time factor (B-) which is 2 stages of extraction, volume ratio had a significant effect on xylitol content after extraction process. This was because during little stages being carried out, the volume ratios became the crucial factor in xylitol extraction.

The Least Significant Difference (LSD) bars act as the visual aids in assisting to interpret effect on interaction plots. As shown in Figure 4.6, the overlapping of the LSD bars for 2 means indicated that both lower coded extraction factor (2 stages) and higher coded extraction factor (6 stages) cover the same range of remaining xylitol mass. In the other words, it defines that the difference in those means is not large enough to be declared significant using a t-test. The overlaps between pairs of LSD bars indicate that the associated means differ is not lie on 95 % confidence levels.

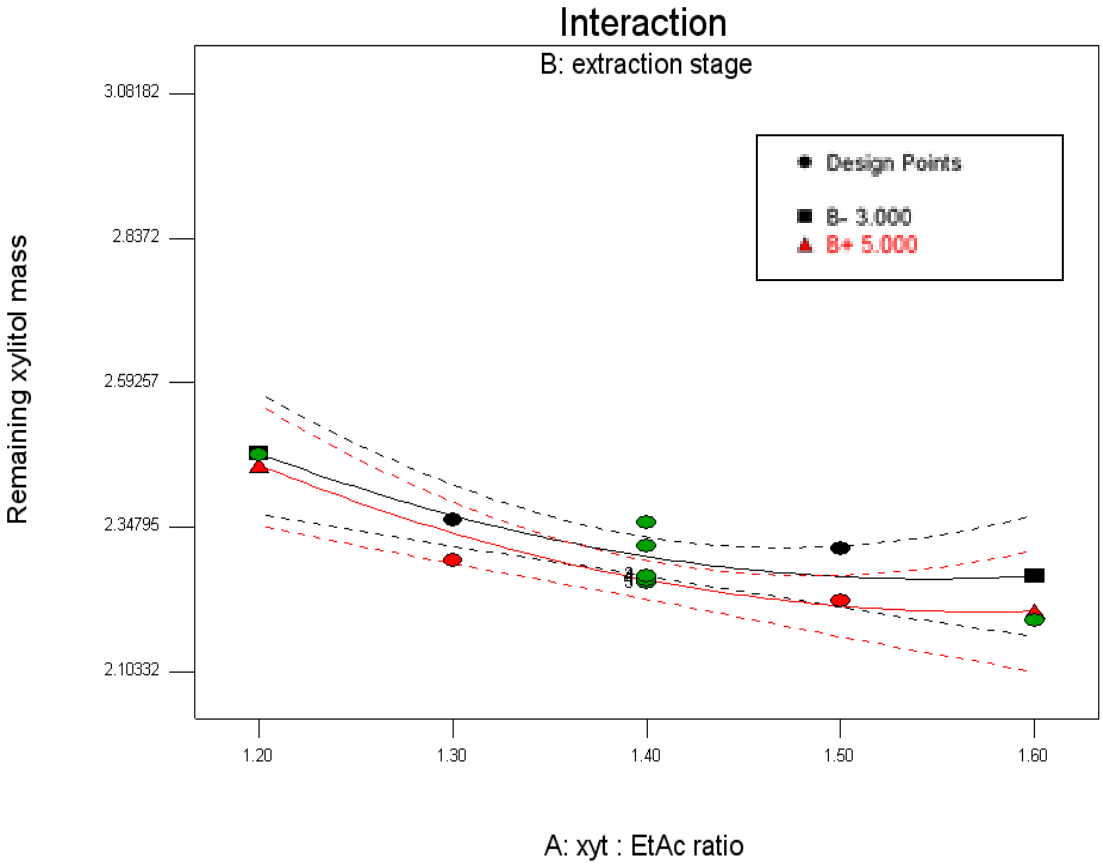


Figure 4.11: Interaction plot of xylitol to Ethyl acetate ratio and extraction stage to remaining xylitol mass.

Table 4.5: Optimization report

Optimizing conditions	Goal	Solutions	
Volume ratios	In range	1:5	
Extraction stages	In range	4.65	**Desirability**
Remaining xylitol content (g)	minimize	2.2122	0.918

Table 4.5 shows the optimization result. With respective limitation conditions for both optimized factors and setting the remaining xylitol mass in aqueous layer as minimum (as desired), two solutions were obtained with 0.918 desirability. Therefore the volume ratio was selected as 1:5 and extraction stage was 5 stages (rounded up) for the validation study and feasibility study from MWS.

4.6 Confirmation test

4.6.1 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 two suggested optimal conditions were obtained. To verify these conditions, xylitol extractions 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. (4.x) and (4.x) outlined by Zularisam et al. (2010), the error in percentage among the actual and predicted values of both responses over a considered ranges of operating variables were calculated.

$$\text{Residual} = (\text{actual value} - \text{predicted value}) \qquad \text{(eq.4.9)}$$

$$\%\ \text{Error} = \frac{Residual}{Actual\ value} \times 100 \qquad \text{(eq.4.10)}$$

The best results of xylitol extraction obtained were 2.21g of xylitol mass remained at the aqueous layer when xylitol to ethyl acetate volume ratios was 1:5 and with 5 number of extraction stages that are typed in boldface in Table 4.9. It is evident that percentage errors ranged from 0.1 to 0.4% for xylitol mass after extraction. These findings indicated that the developed models were sufficiently 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 4.6: Results of verification process with experimental design

Factor 1: Volume ratios	Factor 2: Number of stages	Xylitol mass after extraction			
		Actual**	Predicted	Residual (10^3)	Error (%)
1:5	5*	2.22100	2.21218	8.82	0.4
1:5	5*	2.21400	2.21218	1.82	0.1

**the actual number of stages by RSM is 4.65 however this is not feasible in real practice, thus round up of 5 stages when taken as closest approximation*

***averaged values*

4.6.2 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 (Table 4.xx), the optimum conditions for xylitol extraction were chosen with the xylitol to ethyl acetate volume ratios of 1:5 and 5 number of extracting stages. In these optimized conditions, the model predicted a minimum xylitol mass retained at solution after extraction of 2.212 g, with a possible variation of 2.102 to 2.323 g, at 95% CI. To confirm these results, experiments were performed by employing the model suggested optimum conditions, and the retained xylitol mass (from HPLC analysis) was 2.195 g which was very close to the predicted result, indicating that the models to be reasonably accurate and reliable. Hence, the obtained models could reliably be utilized for the maximum xylitol extraction from commercial prepared xylitol solution.

Table 4.7: Results of confirmation run at the optimum operating conditions

Optimum conditions		Xylitol mass* remained after extraction (g)		
		Actual	**Predicted**	**Error (%)**
Volume ratios	1:5	2.195	2.212	0.8
Stages	5			

**reminder that all the xylitol mass were derived from the HPLC results for each sample*

4.7 Feasibility study result

Feasibility study was conducted in a single run with the synthesized xylitol from Meranti wood sawdust, MWS employing the optimized and validated optimum conditions to extract with ethyl acetate in a separatory funnel.

Table 4.8: Xylitol concentration and yield obtained from MWSHH under conditions optimized by Rafiqul, (2012).

Reaction medium	**Time (h)**	**Xylitol conc. (g/L)**	**Remaining xylose (%)**	**Yield ($Y_{P/S,}$ %)**	**Productivity (Q_p, g/L.h)**
MWSHH (18.8 g/L)	12.25	16.28	13.4	86.6	1.33

Conversion to make up xylitol concentration to 67 g/L as in commercial xylitol solution extractions, 150mL of 16.28 g/L MWSHH was taken and mixed with 740mL of ethyl acetate. Total volume of 890mL with volume ratios of 1:5 and 5 extraction stages was carried out in a 1 Liter separatory funnel. The result was presented in Table 4.8

5 CONCLUSION & RECOMMENDATION

This final chapter is written to summarize all the results and discussion of the data presented in chapter 4. Recommendation for further study is also suggested for xylitol purification using LLE.

5.1 Conclusion

Result and discussion for the HPLC analysis revealed that the xylitol concentration can be quantified and OFAT results that the extraction time is not a significant factor to the extraction of xylitol and the range of significance for factor A: volume ratios of xylitol to ethyl acetate were 1:2 to 1:6 meanwhile the range of significance for factor B: extraction stage were 2 to 6 stages. Further study using CCD in RSM had revealed that the optimization correlation of these factors and their effects to the response. Ultimately, a volume ratio of 1:5 and 5 stages were obtained that promoted the highest extraction yield for xylitol LLE.

In addition, the validation and feasibility studies appeared to be promising as their corresponded to the commercial xylitol results. From this study, we can conclude that the extraction of xylitol from fermented broth using LLE can be improved by 8% after optimizing conditions of extraction: volume ratios and extraction stages. This result correlate with the hypothesis from Mussatto et al.,2006 that optimizing the extraction of xylitol using LLE and ethyl acetate as solvent can enhance the purify efficiency . Besides that, the optimization of xylitol concentration after extraction in this study suggest that the extraction of xylitol to the solvent could be maximized i.e. 0.587 g/g, i.e. 58.77% by applying a 1:5 volumetric ratio of xylitol to ethyl acetate and carried out in a extraction of 5 stages. Essentially, this research has proved the feasibility of purifying xylitol via LLE in lab scale. Therefore, from this study, we can conclude that by using LLE which are be able to purify xylitol from fermented broth in industrial scale downstream processing.

5.2 Recommendation

Nevertheless, further study should be conducted including optimizing the condition for distillation process to recover the xylitol in the solvent after extraction. Besides, addition of optimising parameter e.g. temperature during extraction, different type of extraction, employing continuous extraction are also recommended.

5.3 Future work

The research carried in this project to purify xylitol from fermentation of could add great value to the global sugar-free industrial process in cost cutting and energy saving. It is recommended to construct a pilot study of scale-up experiment for the optimization of xylitol purification via LLE using optimum conditions obtained from this study. Further studies are also required to more thoroughly assess product quality than was done in this work.

A paradigm shift and a more holistic downstream purification framework model that considers energy, products and wastes will make it more sustainable. Besides, a combination of different technologies can be implemented in future research on xylitol purification from fermented broth. As to fulfil the concept of waste-to-wealth, xylitol production research that utilizes waste and non-food energy crops should be focused especially exploring raw materials that are abundantly available in Malaysia.

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APPENDICES

Appendix A

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==========================================================================
                          Calibration Table
==========================================================================



Calib. Data Modified  :      Wednesday, October 15, 2014 9:38:39 AM


Rel. Reference Window :      5.000 %
Abs. Reference Window :      0.000 min
Rel. Non-ref. Window  :      5.000 %
Abs. Non-ref. Window  :      0.000 min
Uncalibrated Peaks    :      not reported
Partial Calibration   :      Yes, identified peaks are recalibrated
Correct All Ret. Times:      No, only for identified peaks

Curve Type            :      Linear
Origin                :      Included
Weight                :      Equal

Recalibration Settings:
Average Response      :      Average all calibrations
Average Retention Time:      Floating Average New 75%

Calibration Report Options :
    Printout of recalibrations within a sequence:
        Calibration Table after Recalibration
        Normal Report after Recalibration
    If the sequence is done with bracketing:
        Results of first cycle (ending previous bracket)

Signal 1: RID1 A, Refractive Index Signal

RetTime   Lvl   Amount      Area     Amt/Area  Ref Grp Name
 [min] Sig     [ng/ul]
-------|--|--|----------|----------|----------|---|--|---------------
 11.808  1  1    1.00000 2.89347e5  3.45605e-6
            2    2.00000 7.19706e5  2.77891e-6
            3    4.00000 1.15877e6  3.45193e-6
            4    6.00000 1.53102e6  3.91895e-6
            5    8.00000 2.31890e6  3.44991e-6
```

Figure A1: Standard curve data of xylitol

Study Type	Response Surface	Runs	13
Initial Design	Central Composite	Blocks	No Blocks
Design Model	Quadratic		

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	xyt : EtAc ratio	v/v	Numeric	1.30	1.50	-1.000	1.000	1.400	0.096
B	extraction stage		Numeric	3.00	5.00	-1.000	1.000	4.000	0.961

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans
Y1	Remaining xylito	g	13	Polynomial	2.189	2.471	2.29331	0.0722142	1.12883	None

Figure A2: CCD design summary using Design Expert V 7.1.6.

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	68.37	1	68.37			
Linear vs Mean	0.043	2	0.022	11.38	0.0026	Suggested
2FI vs Linear	1.000E-004	1	1.000E-004	0.047	0.8326	
Quadratic vs 2FI	0.012	2	6.052E-003	6.15	0.0288	Suggested
Cubic vs Quadra	6.803E-003	2	3.402E-003	188.33	< 0.0001	Aliased
Residual	9.031E-005	5	1.806E-005			
Total	68.43	13	5.26			

*"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	0.019	6	3.169E-003	147.38	0.0001	Suggested
2FI	0.019	5	3.782E-003	175.93	< 0.0001	
Quadratic	6.808E-003	3	2.269E-003	105.54	0.0003	Suggested
Cubic	4.310E-006	1	4.310E-006	0.20	0.6775	Aliased
Pure Error	8.600E-005	4	2.150E-005			

*"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Figure A3: Model fit summary generated using Design Expert V 7.1.6.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.056	5	0.011	11.31	0.0030	significant
A-xyt : EtAc rai	*0.039*	*1*	*0.039*	*39.36*	*0.0004*	
B-extraction st	*4.720E-003*	*1*	*4.720E-003*	*4.79*	*0.0647*	
AB	*1.000E-004*	*1*	*1.000E-004*	*0.10*	*0.7593*	
A^2	*7.237E-003*	*1*	*7.237E-003*	*7.35*	*0.0302*	
B^2	*8.291E-003*	*1*	*8.291E-003*	*8.42*	*0.0229*	
Residual	6.894E-003	7	9.848E-004			
Lack of Fit	*6.808E-003*	*3*	*2.269E-003*	*105.54*	*0.0003*	*significant*
Pure Error	*8.600E-005*	*4*	*2.150E-005*			
Cor Total	0.063	12				

Figure A4: ANOVA test summary table in RSM.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
xyt : EtAc ratio	is in range	1.3	1.5	1	1
extraction stage	is in range	3	5	1	1
Remaining xylito	minimize	2.189	2.471	1	1

Solutions

Number	xyt : EtAc ratio	extraction stage	Remaining xyli	Desirability	
1	1.50	4.65	2.21218	0.918	Selected
2	1.50	4.66	2.21218	0.918	

Figure A5: Optimization solution suggested from Design Expert V 7.1.6.

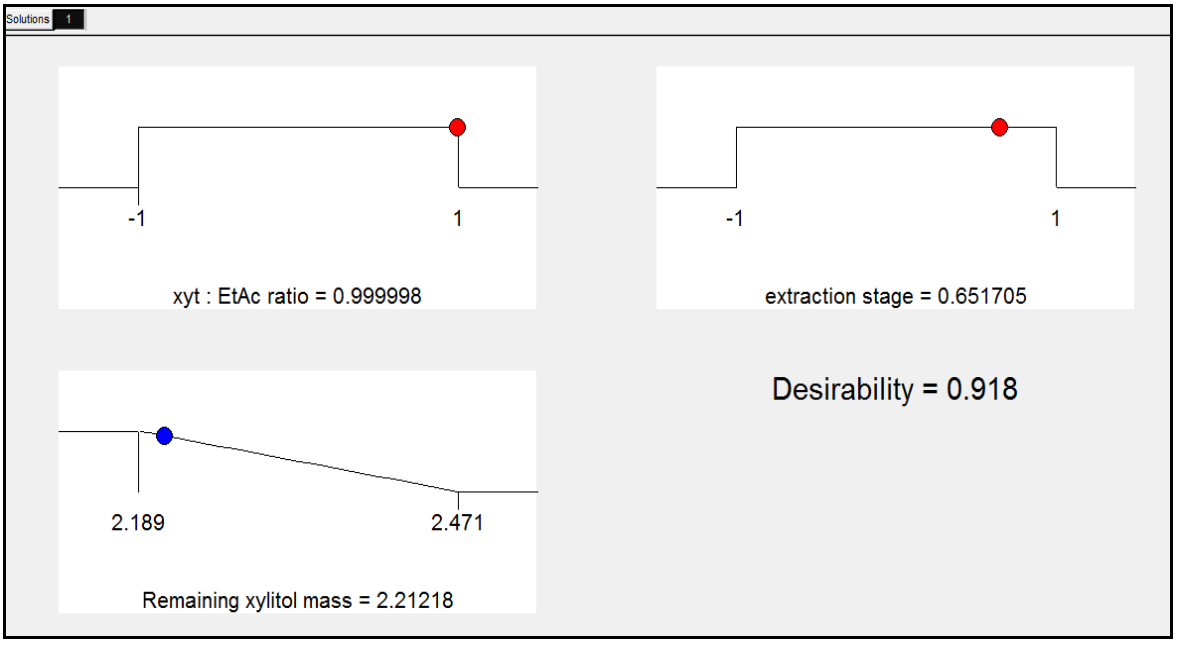

Figure A6: Ramps for parameters and response using Design Expert V 7.1.6.

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	xyt : EtAc ratio	1.40	1.30	1.50	0.000	Actual
B	extraction stage	4.00	3.00	5.00	0.000	Actual

Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Remaining xylitol mass	2.25934	0.013	2.23	2.29	0.034	2.18	2.34

Figure A7: Point prediction from suggested solution in Design Expert V 7.1.6.

Std. Dev.	0.031	R-Squared	0.8898
Mean	2.29	Adj R-Squared	0.8112
C.V. %	1.37	Pred R-Squared	-0.1094
PRESS	0.069	Adeq Precision	10.771

Figure A8: R-squared values from in Design Expert V 7.1.6.

Appendix B

Table B-1: OFAT (Factor A)

v/v	runs	nRIU	conc.	actual conc.(g/L)	v (L)	m(g)	x2	x1	x1-x2	% extracted	% retained in AQ
1:1	1	1.92E+06	6.8291021	68.29102101	0.108	7.37543	7.645168	12.06	4.414832	54.91084844	45.08915156
	2	1.98E+06	7.05201092	70.52010916	0.108	7.616172		0	0		
	3	2.06E+06	7.35546416	73.55464165	0.108	7.943901		0	0		
1:3	1	1.97E+06	7.01598267	70.15982673	0.054	3.788631	3.809391	6.03	2.220609	55.23903533	44.76096467
	2	1.96E+06	6.98221303	69.82213026	0.054	3.770395		0	0		
	3	2.01E+06	7.16508641	71.65086412	0.054	3.869147		0	0		
1:5	1	1.93E+06	6.89237921	68.92379207	0.036	2.481257	2.49041	4.02	1.52959	57.07423941	42.92576059
	2	1.90E+06	6.7543863	67.54386301	0.036	2.431579		0	0		
	3	1.99E+06	7.10665436	71.06654358	0.036	2.558396		0	0		
1:7	1	2.04E+06	7.27750619	72.77506187	0.027	1.964927	1.974218	3.015	1.040782	51.78018699	48.21981301
	2	1.98E+06	7.07681904	70.76819039	0.027	1.910741		0	0		
	3	2.12E+06	7.58143301	75.81433013	0.027	2.046987		0	0		
1:11	1	2.06E+06	7.35316914	73.5316914	0.018	1.32357	1.338569	2.01	0.671431	50.10679274	49.89320726
	2	2.00E+06	7.14359332	71.43593325	0.018	1.285847		0	0		
	3	2.19E+06	7.81272049	78.12720489	0.018	1.40629					

Table B-2: OFAT (Factor B)

no.stages	runs	nRIU	conc.	actual conc. (g/L)	v(L)	m(g)	x	std
1	1	1.96E+06	6.98742237	69.87422367	0.07	4.891196	4.978373	0.113453
	2	2.04E+06	7.29521066	72.95210663	0.07	5.106647		
	3	1.98E+06	7.0532495	70.532495	0.07	4.937275		
2	1	1.90E+06	6.77846584	67.78465844	0.06	4.06708	4.414131	0.300626
	2	2.14E+06	7.63520216	76.3520216	0.06	4.581121		
	3	2.14E+06	7.65698668	76.56986679	0.06	4.594192		
3	1	1.90E+06	6.74990554	67.49905538	0.05	3.374953	3.658375	0.327948
	2	2.50E+06	8.95648059	89.56480592	0.04	3.582592		
	3	2.49E+06	8.92795672	89.27956715	0.045	4.017581		
4	1	2.97E+06	10.6567295	106.5672951	0.04	4.262692	3.462242	0.766651
	2	2.54E+06	9.11527444	91.15274435	0.03	2.734582		
	3	2.70E+06	9.6841491	96.84149098	0.035	3.389452		
5	1	3.19E+06	11.4530302	114.5303018	0.03	3.435909	3.232716	0.264712
	2	3.26E+06	11.7334603	117.3346032	0.025	2.933365		
	3	3.09E+06	11.0962449	110.9624493	0.03	3.328873		
6	1	3.09E+06	11.0994143	110.9941425	0.027	2.996842	3.004584	0.499197
	2	3.49E+06	12.5465183	125.4651832	0.02	2.509304		
	3	3.36E+06	12.0951999	120.9519991	0.029	3.507608		
7	1	3.32E+06	11.9481726	119.4817262	0.023	2.74808	2.810339	0.567267
	2	3.72E+06	13.3927631	133.9276309	0.017	2.27677		
	3	3.78E+06	13.6246699	136.2466986	0.025	3.406167		
8	1	3.90E+06	14.0699046	140.6990463	0.02	2.813981	2.636109	0.591816
	2	3.66E+06	13.1717122	131.7171215	0.015	1.975757		
	3	4.12E+06	14.8504316	148.5043155	0.021	3.118591		
9	1	4.63E+06	16.7214959	167.2149587	0.017	2.842654	2.631335	0.229369

	2	5.08E+06	18.3646606	183.6466061	0.013	2.387406
	3	4.34E+06	15.6702653	156.7026533	0.017	2.663945

Table B-3: RSM suggested model runs

21-Nov	Run	replicates	F1 : v/v	F2 : N-staging	area (y)	amount(x)	dilution x10	Avr (x)	final V(L) at n-stage	m (g)
	1	1	1 to 2	4	2.53E+06	9.047115848	90.47115848	77.90013	0.0315	2.453854
		2	1 to 2	4	2.04E+06	7.287378436	72.87378436		0.0315	0
		3	1 to 2	4	1.97E+06	7.035545025	70.35545025		0.0315	0
	2	1	1 to 4	4	2.87E+06	10.31116438	103.1116438	103.6083	0.021	2.175774
		2	1 to 4	4	3.00E+06	10.76798356	107.6798356		0.021	0
		3	1 to 4	4	2.79E+06	10.00333966	100.0333966		0.021	0
	3	1	1 to 4	4	2.79E+06	10.01026116	100.1026116	109.8815	0.0205	2.25257
		2	1 to 4	4	2.97E+06	10.67563614	106.7563614		0.021	0
		3	1 to 4	4	3.41E+06	12.27854688	122.7854688		0.021	0
	4	1	1 to 3	5	2.69E+06	9.638030032	96.38030032	98.65662	0.022	2.170446
		2	1 to 3	5	2.78E+06	9.979478688	99.79478688		0.028	0
		3	1 to 3	5	2.78E+06	9.979478688	99.79478688		0.028	0
	5	1	1 to 4	6	3.19E+06	11.4828655	114.828655	128.9941	0.016	2.063905
		2	1 to 4	6	3.62E+06	13.03823644	130.3823644		0.016	0
		3	1 to 4	6	3.93E+06	14.17711506	141.7711506		0.016	0
22-Nov	6	1	1 to 4	4	2.95E+06	10.61104759	106.1104759	104.7777	0.0215	2.25272
		2	1 to 4	4	2.76E+06	9.905564325	99.05564325		0.0215	0
		3	1 to 4	4	3.04E+06	10.91668658	109.1668658		0.0215	0
	7	1	1 to 4	4	3.36E+06	12.08274121	105.827401	112.1695	0.0205	2.299474
		2	1 to 4	4	3.20E+06	11.49918567	114.9918567		0.024	0
		3	1 to 4	4	3.22E+06	11.56891071	115.6891071		0.024	0
	8	1	1 to 6	4	3.85E+06	13.88091117	138.8091117	144.5671	0.0155	2.24079
		2	1 to 6	4	4.11E+06	14.80850182	148.0850182		0.015	0
		3	1 to 6	4	4.07E+06	14.68070902	146.8070902		0.015	0
	9	1	1 to 5	5	4.29E+06	15.46090809	154.6090809	151.0395	0.015	2.265593

	2	1 to 5	5	4.23E+06	15.25384587	152.5384587		0.015	0
	3	1 to 5	5	4.05E+06	14.59710456	145.9710456		0.015	0
10	1	1 to 4	2	1.91E+06	6.813619792	68.13619792	79.15317	0.033	2.612054
	2	1 to 4	2	2.44E+06	8.720821641	87.20821641		0.033	0
	3	1 to 4	2	2.30E+06	8.21150833	82.1150833		0.033	0
11	1	1 to 3	3	2.71E+06	9.700797133	97.00797133	91.52918	0.026	2.379759
	2	1 to 3	3	2.44E+06	8.730803176	87.30803176		0.026	0
	3	1 to 3	3	2.52E+06	9.027152777	90.27152777		0.026	0
12	1	1 to 5	3	2.95E+06	10.58460017	105.8460017	103.5156	0.0235	2.432618
	2	1 to 5	3	2.82E+06	10.1030093	101.030093		0.0235	0
	3	1 to 5	3	2.89E+06	10.36708284	103.6708284		0.0235	0
13	1	1 to 4	4	3.35E+06	12.04186791	120.4186791	105.0587	0.021	2.206232
	2	1 to 4	4	2.80E+06	10.05350234	100.5350234		0.021	0
	3	1 to 4	4	2.63E+06	9.422224865	94.22224865		0.021	0

*Initial mass = 5.28 g