ISOLATION AND IDENTIFICATION OF MICROORGANISMS FOR XYLITOL PRODUCTION FROM LIGNOCELLULOSIC HYDROLYSATES

PARAMEE WADDUANGDEE

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biotechnology
Suranaree University of Technology
Academic Year 2022

การคัดแยกและการคัดเลือกสายพันธุ์จุลินทรีย์เพื่อใช้ผลิตไซลิทอล จากลิกโนเซลลูโลสไฮโดรไลเสส

นางสาวปรมี หวาดด้วงดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

ISOLATION AND IDENTIFICATION OF MICROORGANISMS FOR XYLITOL PRODUCTION FROM LIGNOCELLULOSIC HYDROLYSATES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Panlada Tittabutr)
Chairperson

(Assoc. Prof. Dr. Apichat Boontawan)
Member (Thesis Advisor)

(Assoc. Prof. Dr. Mariena Ketudat-Cairns)
Member

(Assoc. Prof. Dr. Prakit Sukyai)
Member

(Assoc. Prof. Dr. Chatchai Jothityangkoon)
Vice Rector for Academic Affairs
and Quality Assurance

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural
Technology

ปรมี หวาดด้วงดี: การคัดแยกและการคัดเลือกสายพันธุ์จุลินทรีย์เพื่อใช้ผลิตไซลิทอลจากลิกโนเซลลูโลสไฮโดรไลเสส (ISOLATION AND IDENTIFICATION OF MICROORGANISMS FOR XYLITOL PRODUCTION FROM LIGNOCELLULOSIC HYDROLYSATES)
อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.อภิชาติ บุญทาวัน, 61 หน้า.

คำสำคัญ: ไซลิทอล/กากมะพร้าวไฮโดรไลเลส/*Meyerozyma guilliermondii*

กระบวนการผลิตไซลิทอลทางชีวภาพ เป็นวิธีการที่เป็นมิตรต่อสิ่งแวดล้อมและง่ายต่อการดำเนินงานเมื่อเทียบกับกระบวนการทางเคมี การศึกษาก่อนหน้านี้มักจะผลิตไซลิทอลจากยีสต์เนื่องจากอัตราการผลิตไซลิทอลสูงกว่าเมื่อเทียบกับจุลินทรีย์ชนิดอื่นงานวิจัยฉบับนี้มีวัตถุประสงค์เพื่อคัดแยกสายพันธุ์จุลินทรีย์ที่ผลิตไซลิทอลจากแหล่งต่าง ๆ ในประเทศไทย โดยใช้อาหารที่เหมาะสมต่อการผลิตไซลิทอลในการคัดแยกและการคัดเลือกจุลินทรีย์ ได้แก่ ไซโลสและเปปโทน เพื่อศึกษาความสามารถในการใช้ไซโลสในจุลินทรีย์ งานวิจัยนี้สามารถคัดแยกและคัดเลือกจุลินทรีย์ได้ทั้งหมด 36 สายพันธุ์ จากนั้นทำการวิเคราะห์อัตราการผลิตไซลิทอลของจุลินทรีย์แต่ละชนิด และเลือก 10 อันดับที่ผลผลิตไซลิทอลสูงที่สุด เพื่อทำการระบุสายพันธุ์โดยการหาลำดับนิวคลีโอไทป์ของ 16S rDNA และ 26S rDNA ผลการทดลองพบจุลินทรีย์ 3 สายพันธุ์ ได้แก่ *Candida tropicalis*, *Meyerozyma carpophila* และ *Meyerozyma guilliermondii* หลังจากนั้นทำการวิเคราะห์พารามิเตอร์ที่ใช้ในการปรับสภาพกากมะพร้าว ได้แก่ อัตราส่วนของแข็งและของเหลว, ความเข้มข้นกรด, อุณหภูมิ, และเวลา พบว่าอัตราส่วนของแข็งและของเหลวร้อยละ 12 กรดซัลฟิวริกเจือจางร้อยละ 9 ที่ 120 °C เป็นเวลา 20 นาที จะทำให้ได้ไซโลสที่ความเข้มข้นสูงที่สุดประมาณ 28.65 กรัมต่อลิตร จากนั้นกำจัดสารยับยั้งการผลิตไซลิทอลที่เกิดขึ้นโดยใช้ถ่านกัมมันต์ร้อยละ 3 เป็นเวลา 1 ชั่วโมง จากเปรียบเทียบการเพาะเลี้ยง *M. guilliermondii* C511C ด้วยกากมะพร้าวไฮโดรไลเสสที่ผ่านการกำจัดสารยับยั้งและไม่ผ่านการกำจัดสารยับยั้ง เชื้อนี้สามารถผลิตไซลิทอลได้ 14.61 ± 0.10 และ 12.50 ± 0.01 กรัมต่อลิตร ตามลำดับ การหมักแบบกึ่งกะในถังปฏิกรณ์ชีวภาพ 5 ลิตรด้วยกากมะพร้าวไฮโดรไลเสสที่ผ่านการกำจัดสารยับยั้ง โดยอาหารที่ประกอบด้วยสารสกัดยีสต์ 3 กรัมต่อลิตร, KH_2PO_4 2 กรัมต่อลิตร, กลูโคส 5 กรัมต่อลิตรและ $(NH_4)_2SO_4$ 2 กรัมต่อลิตร ที่อุณหภูมิ 30 °C, pH 5.5, และการกวนที่อัตราเร็ว 300 rpm แสดงให้เห็นถึงศักยภาพในการผลิตไซลิทอลของ *M. guilliermondii* C511C หลังจาก 96 ชั่วโมงของการหมัก พบว่าสามารถผลิตไซลิทอลได้ 28.19 กรัมต่อลิตร ด้วยอัตราการผลิต 0.70 กรัมต่อกรัม เมื่อเทียบกับกระบวนการทางเคมีและพบว่ากระบวนการทางชีวภาพมีประสิทธิภาพในการผลิตไซลิทอลได้มากกว่ารวมทั้งความบริสุทธิ์ของไซลิทอลที่สูงกว่า

M. guilliermondii C511C แสดงให้เห็นถึงประสิทธิภาพในการผลิตไซลิทอลสูง รวมทั้งเป็นยีสต์ที่ไม่ก่อโรค ซึ่งคุณสมบัติเช่นนี้มีความสำคัญอย่างยิ่งในด้านความปลอดภัยในการใช้งาน เนื่องจากทำให้มั่นใจได้ว่ากระบวนการผลิตจะไม่ก่อให้เกิดความเสี่ยงในด้านสุขภาพต่อบุคลากรที่เกี่ยวข้องในกระบวนการ รวมทั้ง *M. guilliermondii* C511C มีความสามารถในการใช้เฮมิเซลลูโลสไฮโดรไลเสตจากกากมะพร้าวอ่อน ซึ่งเป็นการเพิ่มมูลค่าให้กับของเหลือทิ้งจากอุตสาหกรรมการเกษตรโดยเฉพาะกากมะพร้าวได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PARAMEE WADDUANGDEE: ISOLATION AND IDENTIFICATION OF MICROORGANISMS FOR XYLITOL PRODUCTION FROM LIGNOCELLULOSIC HYDROLYSATES. THESIS ADVISOR: ASSOC. PROF. APICHAT BOONTAWAN, Ph.D., 61 PP.

Keyword: Xylitol/Coconut husk hydrolysate/*Meyerozyma guilliermondii*

The biotechnological production of xylitol has gained significant attention as an environmentally friendly and easily manageable alternative to chemical production methods. In this current work, the focus was on isolating xylitol-producing yeast strains from various sources in Thailand. The rationale behind using yeast in xylitol production lies in its ability to achieve high yields, making it a preferred choice for many previous studies. The screening process involved isolating a total of 36 yeast strains and subjecting them to xylitol production tests using enriched medium containing xylose and peptone. The outcome of this screening led to the identification of 10 yeast strains that exhibited the best xylitol production capabilities. Further characterization of these strains revealed that they belonged to three species: *Candida tropicalis*, *Meyerozyma carpophila*, and *Meyerozyma guilliermondii*. To optimize the xylitol production process, various parameters of pretreatment were investigated. These included solid and liquid ratios, acid concentrations, residence temperature, and time. The results showed that by pretreating green coconut husk (GCH) with 9% dilute H_2SO_4 at 120 ℃ for 20 min, a maximum xylose concentration of 28.65 g/L was achieved from 12% GCH. To ensure efficient xylitol production, the GCH hydrolysate underwent detoxification with 3% activated carbon for 1 h. The subsequent fermentation process in a 5-L bioreactor using the fed-batch mode demonstrated the high potential of *M. guilliermondii* C511C for xylitol production. It yielded an impressive 28.19 ± 0.46 g/L of xylitol with a yield of 0.70 g/g when supplemented with 3 g/L yeast extract, 2 g/L KH_2PO_4, 5 g/L glucose, and 2 g/L $(NH_4)_2SO_4$ at 30 ℃, pH 5.5, and 300 rpm of agitation after 96 hours of fermentation time. Comparing this biotechnological approach with chemical processes, it become evident that the former offers significant advantages. The simplified fed-batch fermentation process for xylitol production from xylose-rich hydrolysate not only yields considerable quantities of xylitol but also avoids the complexities and environmental hazards associated with chemical methods.

Furthermore, the use of *M. guilliermondii* C511C, which demonstrated excellent xylitol production capabilities, is noteworthy for its non-pathogenic nature. This characteristic is particularly important from a safety standpoint, as it ensures that the production process does not pose any health risks to the personnel involved.

Additionally, the modified process developed in this study, optimized for upscaling, presents a promising solution to mitigate the pollution problem caused by green coconut husk in the beverage industry. By utilizing green coconut husk as a carbon source for xylitol production, this work offers an innovative approach to valorize agricultural waste and reduce its environmental impact.

In conclusion, the results of this study showcase the high potential of biotechnological xylitol production using *M. guilliermondii* C511C. The simplicity of the fed-batch fermentation process, the non-pathogenic nature of the selected yeast strain, and the effective utilization of agricultural waste as a feedstock underscore the significance of this research in sustainable bioprocess engineering. The findings of this study hold promise for the development of eco-friendly and economically viable xylitol production methods, contributing to a greener and more sustainable future. However, further research and optimization studies are encouraged to explore the full potential of this approach and facilitate its integration into industrial applications.

School of Biotechnology Student's Signature _____

Academic Year 2022 Advisor's Signature _____

ACKNOWLEDGEMENTS

I would like to express my heartfelt appreciation to all those who have contributed to the completion of this thesis. I am immensely grateful to my thesis advisor, Assoc. Prof. Dr. Apichat Boontawan, for his exceptional guidance, valuable advice, unwavering support, and various forms of assistance throughout this study, which have greatly enriched my graduate experience.

I would also like to extend a special thanks to my co-advisor, Assoc. Prof. Dr. Mariena Ketudat-Cairns, for her supervision and suggestion for intriguing topics for this experiment. Your constant support and guidance have been invaluable to our research group.

A special thanks goes to Dr. Sumeth Imsoonthornruksa for providing me with training and sharing your extensive knowledge. I have not only gained profound scientific insights from you, but also valuable practical experience that you generously shared with me.

Additionally, I would also like to express my sincere appreciation to Assoc. Prof. Dr. Panlada Tittabutr and Assoc. Prof. Dr. Prakit Sukyai for dedicating their valuable time to serve as members of my thesis defense committee. Your feedback and constructive criticism have been instrumental in improving the quality of my work.

Furthermore, I am grateful to the faculty staff, friends at the School of Biotechnology, as well as the members of AB's Laboratory, for their generosity in providing me with equipment, valuable information on biotechnology, and their kind assistance throughout my research journey.

Paramee Wadduangdee

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

°C	=	Degree Celsius
%	=	Percent
w/w	=	Weight per Weight
v/v	=	Volume per Volume
w/v	=	Weight per Volume
g/L	=	Gram per Liter
g/L.h	=	Gram per Liter per Hour
g	=	Gram
s	=	Second
g/g	=	Gram per Gram
mL/min	=	Milliliter per Minute
mM	=	Millimolar
g	=	G-force
L	=	Liter
mL	=	Milliliter
rpm	=	Round per Minute
vvm	=	Volume per Volume per Liter
min	=	Minute
h	=	Hour
et al.,	=	and other
OD_{600}	=	Optical density measured at 600 nm
bp	=	Base pairs
DI	=	Deionized water
XP	=	Xylose and peptone
µL	=	Microliters
µm	=	Micrometer
DOE	=	Design of experiment
Y_x	=	Xylitol yield from xylose (g/g)

LIST OF ABBREVIATIONS (Continued)

GCH	=	Green coconut husk
SCB	=	Sugarcane bagasse
AC	=	Activated carbon
HPLC	=	High Performance Liquid Chromatography
RSM	=	The response surface methodology
YPD	=	Yeast extract, peptone, and dextrose medium
TLC	=	Thin layer chromatography
sp.	=	Species
ITS	=	Internal transcribed spacer

CHAPTER 1
INTRODUCTION

1.1 Significant of this study

Xylitol, a natural sugar alcohol with five carbons, holds significant value as a microorganism product and finds widespread application as a sweetener in industries such as food, nutraceuticals, beverages, and pharmaceuticals. Its global importance as a bioproduct ranks it among the top 12. Xylitol is classified as "Generally Recognized as Safe" (GRAS) by the Food and Drug Administration (FDA) and is utilized as a low-calorie sweetener in more than 35 countries. Its unique pharmacological properties make it useful for treating various disorders.

Xylitol can be produced through both chemical and biotechnological methods. Chemical processes offer high yields (around 90%) and purity at a low cost but require the use of toxic chemicals, high temperatures, and pressures. Biotechnological methods, on the other hand, yield lower purity (ranging from 40% to 80%) but are environmentally friendly and easier to handle compared to chemical methods.

Despite the growing global demand for xylitol, its production remains more expensive than that of other sugars like sucrose and glucose. Lignocellulosic materials, such as straw, stover, and bagasse, represent abundant yet underutilized bio-resources worldwide. These materials are typically considered waste from industrial, agricultural, and forestry activities involving plant biomass (de Freitas Branco et al., 2011). However, they possess a rich potential as a starting point for commercial bioprocessing. Lignocellulosic materials consist of lignin, cellulose, and hemicellulose, with hemicellulose comprising five sugar monomers, including D-xylose, L-arabinose, and D- glucose (Ravindran & Jaiswal, 2016). Sugarcane bagasse, a biomass generated during sugarcane processing, is widely available globally and has been extensively studied for energy and environmental sustainability purposes. To extract monomeric sugars from lignocellulosic materials, pretreatment techniques are crucial. Alkaline, alkali -acid, and acid processes have shown effectiveness in achieving successful hydrolysis

(Ajala et al., 2021). Diluted sulfuric acid combined with high temperature is a common pretreatment method, but it requires neutralization before fermentation and has potential drawbacks such as lignin-related matrix structures and the generation of degradation products (Chen et al., 2022). This study focuses on the pretreatment of lignocellulosic materials using diluted sulfuric acid at high temperature.

The biotechnological process involves the use of bacteria, yeast, and fungi to convert xylose to xylitol. *Candida* and *Debaromyces* are the two most prolific xylitol-producing microorganisms, with *Candida* sp. demonstrating a yield of approximately 0.90 g/g (Kaur et al., 2022). However, some *Candida* species, including C. *tropicalis*, are human pathogens (Yu et al., 2022). Hence, this study concentrates on non-human pathogenic microorganisms, such as *Pichia* sp. and *Saccharomyces* sp.

In this study, microorganisms were isolated and screened from diverse sources in Thailand, including soil, sugarcane bagasse, rice, coconut husk, and corn, to evaluate their capacity for xylitol production. Lignocellulosic biomass hydrolysates derived from sugarcane bagasse and green coconut husk were employed as carbon sources for microbial growth. Furthermore, the study explored the optimization of xylitol production using a 5-L bioreactor. The outcomes of this research are expected to contribute to the utilization of lignocellulosic biomass and advance the agricultural and sugar-alcohol sectors.

1.2 Research objective

The main objectives of this study were to isolate and identify xylitol-producing microorganisms from different sources in Thailand, as well as to investigate the suitable fermentation parameters for enhancing xylitol production through fed-batch fermentation. Additionally, the study aimed to explore the optimal pretreatment parameters for lignocellulose hydrolysate to achieve a high concentration of xylose. Furthermore, the research focused on expanding xylitol production capacity in a 5-L bioreactor by utilizing a high-potential microorganism with lignocellulosic hydrolysates. Thus, this work has focused on:

1. To isolate, characterize, and identify the microorganisms for xylitol production from various sources in Thailand.
2. To determine the optimal conditions for lignocellulosic biomass pretreatment to achieve a high concentration of xylose.
3. To Scaling up xylitol production in a 5-L bioreactor using the identified high-potential microorganism and lignocellulose hydrolysates.

1.3 Scope and limitation of the thesis

The study focuses on the isolation and characterization of microorganisms from various environmental sources, including soil, bagasse, corn, rice, sugarcane, and coconut. Through rDNA and ITS sequencing, the research aims to identify the most effective strain capable of producing xylitol. Additionally, the study evaluates xylitol production utilizing lignocellulosic biomass hydrolysate as a carbon source. The investigation delves into optimizing parameters for lignocellulose hydrolysate production, exploring different pretreatment methods such as dilute acid treatment and varying temperatures. The development of xylitol production is carried out in a 5-L bioreactor. However, the investigation of lignocellulosic biomass hydrolysate conditions may not encompass all possible optimization parameters, and the selected pretreatment methods may not encompass the entire range of potential options. Moreover, the scale-up of xylitol production is limited to the 5-L bioreactor, which may differ from larger-scale production systems. Lastly, the comparison to conventional xylitol production methods may not encompass all existing conventional approaches. These limitations provide insights into the research's boundaries and indicate potential avenues for further exploration and enhancement.

CHAPTER 2
LITERATURE REVIEW

2.1 Xylitol

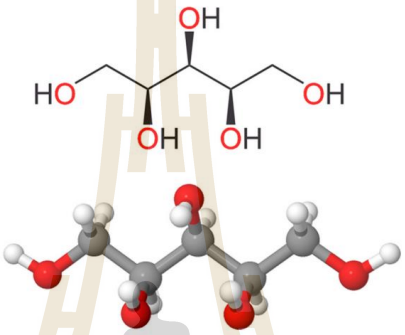

Figure 2.1 The xylitol structure (Gasmi Benahmed et al., 2020).

Sugar alcohols are recognized for affecting blood glucose much less than sucrose. As a result, they are used as sugar alternatives or sweeteners for diabetics. A pentahydroxy alcoholic sugar, xylitol is one of the most valuable microorganism products and is frequently used as a sweetener. One of the top 12 worldwide bio-products, xylitol has a wide range of uses in the food, beverage, nutraceuticals, and pharmaceutical industries. The Food and Drug Administration (FDA) classifies xylitol as a "Generally Recognized as Safe" (GRAS) additive, and more than 35 nations recognize it as a low-calorie sweetener. Xylitol's unique pharmacological capabilities for the treatment of many illnesses are made possible by its less reactive chemical nature. The significant increase in xylitol production is a result of the rising global demand brought on by a fuller understanding of its beneficial characteristics. The production of chewing gum and confectionery goods accounts for over 70% of the market share internationally. The European Food Safety Agency has also asserted that xylitol chewing gum lowers children's caries risk (Salli et al., 2019).

2.1.1 The xylitol application

Xylitol is a naturally occurring sweetener that is high in sweetness, low in energy, and safe for consumption. As a result, xylitol is used widely in daily life and has become the most common kind, particularly in the practical areas of food and medicine (Lugani et al., 2015). Xylitol is generally used as a sweetener in the food industry to produce candies, chocolate, beverages, jams, and snacks; however, it is also employed in adjuvant therapy in the pharmaceutical sector to treat diabetes and dental disease (Mohamad et al., 2015). Numerous studies have shown how xylitol is beneficial for human health, particularly for people who need to follow a diet, are obese, or have diabetes. Additionally, xylitol is frequently used in products to prevent tooth decay, such as toothpaste and sugarless gum, because of its inherent antibacterial characteristics. Ly and colleagues found that xylitol can lower the amount of *Streptococcus* in saliva and tooth plaque, which can prevent cavities. Consumer access to food products containing xylitol that have the purpose of reducing tooth decay is growing (Kiet A. Ly et al., 2006).

In addition to helping to avoid dental diseases, xylitol has also been proven to be an effective treatment for other health-related issues. For instance, xylitol enhances skin functionality and inhibits microbial growth on the skin's surface. Other research suggests that it functions similarly to a prebiotic, which is poorly absorbed in the small intestine and travels to the colon to provide nutrition for microorganisms that aid in better digestion, absorption, and laxative and constipation relief (Gong et al., 2015). There has also been investigation into how xylitol affects people's throat, nose, and ear health. By utilizing xylitol-containing nasal spray twice a day, it has been demonstrated to improve respiratory function in patients with non-allergic nasal congestion (Cingi et al., 2014). When utilizing a 15% xylitol gum solution for three months, the effects of xylitol on sore throat were also demonstrated, leading to enhanced swallowing ability and significantly lessened inflammation (Salli et al., 2019).

2.1.2 The production of xylitol

The transition of commercial production processes to more affordable and environmentally friendly ones marks the beginning of the road toward sustainability. Like biofuels, successive generations of xylitol production techniques can be categorized depending on the sources and catalytic agents employed for substrate modification (Figure 2.2). The extraction of xylitol from plant sources (such as fruits, vegetables, and trees) is followed by the catalytic reduction of xylose in the first two generations, which are traditional and rudimentary techniques of producing xylitol. Due to the need for specialized and expensive equipment, protracted purification processes, catalyst deactivation, and lastly, the significant energy consumption, both

systems have high production costs. Alternative techniques for producing xylitol in a more cost-effective and environmentally friendly way have been devised (Ahuja et al., 2020).

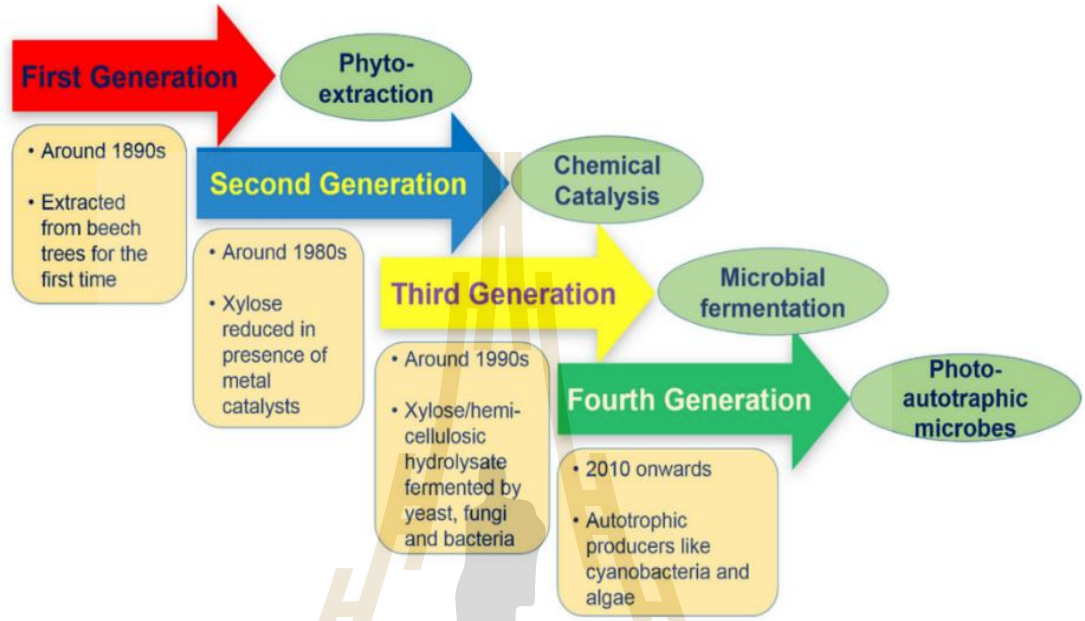

Figure 2.2 Xylitol is produced across several generations using different substrates and catalysts (Ahuja et al., 2020).

2.1.2.1 First generation (Phyto-extraction)

One of the most significant natural sources of xylitol is plant life. Due to a lack of knowledge, xylitol was extracted from wood as the primary and likely only source until the 1960s. Later, it was also recovered by solvent extraction from fruits and vegetables (Ahuja et al., 2020).

2.1.2.2 Second generation (Catalytic reduction)

In Finland, mass production of D-xylose was developed in the 1970s using chromatographic separation from diverse woody hemicelluloses. After production, D-xylose was catalytically reduced to xylitol at high hydrogen pressure and temperature. The need for pure d-xylose feed for catalytic reduction necessitated the use of many purification steps in these processes. In a nutshell, chemical catalysis entails the reduction of xylose at high pressures and temperatures (80-140 °C and 800 psi pressures), all while using a metal catalyst and Raney nickel, a solid catalyst made of tiny grains of a nickel–aluminum alloy. Additionally, additional metal catalysts, such

as ruthenium, titanium, or a combination of different metals, have been applied to catalyze reductions at various temperatures. Depending on the purity of the substrate and the circumstances of the reaction, catalytic reduction produced a conversion of xylose of up to 60% (Hernandez-Mejia et al., 2016). Figure 2.3 compares the production of chemicals and biotechnology.

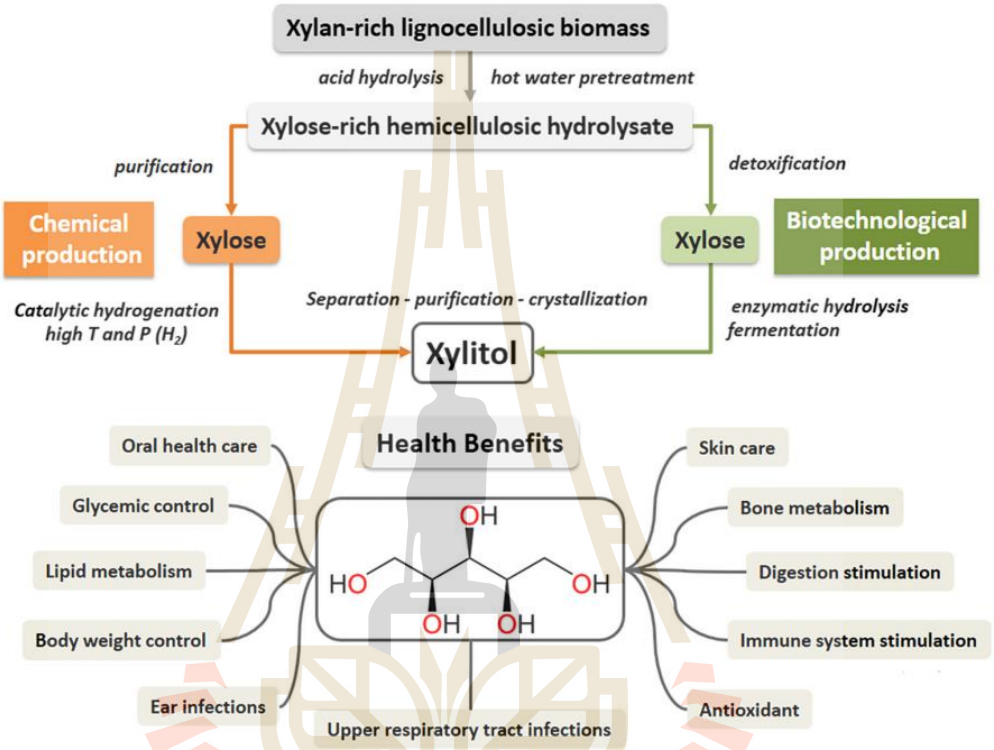

Figure 2.3 Xylitol production and health benefits (Gasmi Benahmed et al., 2020).

2.1.2.3 Third generation (Microbial fermentation)

Microbes naturally manufacture xylitol by converting xylose to xylitol via the xylose reductase enzyme, just like plants do. In natural xylose-using microorganisms, three xylose catabolic pathways have been identified, and metabolic xylose is transported in microbes via two distinct pathways (Figure 2.4).

There are now three known xylose catabolic routes in naturally occurring bacteria that use xylose (Figure 2.4). The first pathway is the XR-XDH pathway, which is frequently found in naturally occurring yeasts that use xylose, including *Saccharomyces stipitis* and *C. shehatae* (Figure 2.4). In this process, xylose is reduced to xylitol by xylose reductase (XR), which is then oxidized to xylulose by xylitol dehydrogenase (XDH). A more direct approach is the XI pathway, which uses xylose

isomerase (XI; Figure 2.4) to transform xylose into xylulose in a single step. From there, xylulose can be directed into glycolysis by phosphorylation and various biochemical processes in the non-oxidative PPP. In several types of fungus and bacteria, the XI route is intrinsic. The Weimberg process, shown in Figure 2.4, is the third xylose metabolic pathway. Xylose is oxidized by xylose dehydrogenase (XylB) to xylono-lactone, which is then transformed to xylonate by xylono-lactone lactonase (XylC). Xylonate then undergoes two sequential dehydration processes by xylonate dehydratase (XylD) and 2-keto-3-deoxy-xylonate dehydratase (XylX) to create α-ketoglutarate semialdehyde, which is further oxidized to α-ketoglutarate by α-ketoglutarate semialdehyde dehydrogenase (XylA) and enters the TCA cycle. Due to its simplicity of expression and high metabolic flux, the XR-XDH route is the most investigated among these pathways in recombinant yeasts. This route has been successfully expressed in non-xylose-fermenting yeasts including *Saccharomyces cerevisiae* and *Y. lipolytica* (Kim et al., 2002; Wang et al., 2021). In contrast to *S. cerevisiae*, where the XI system obtained from *Piromyces* and other organisms functions well, the functional production of the bacterial XI route in non-xylose fermenting yeasts is difficult (Kavya & Nadumane, 2023). The Weimberg route, which has only recently been established in *S. cerevisiae* with, although, low efficiency of xylose metabolism, is significantly more difficult to introduce (Bevilaqua et al., 2023).

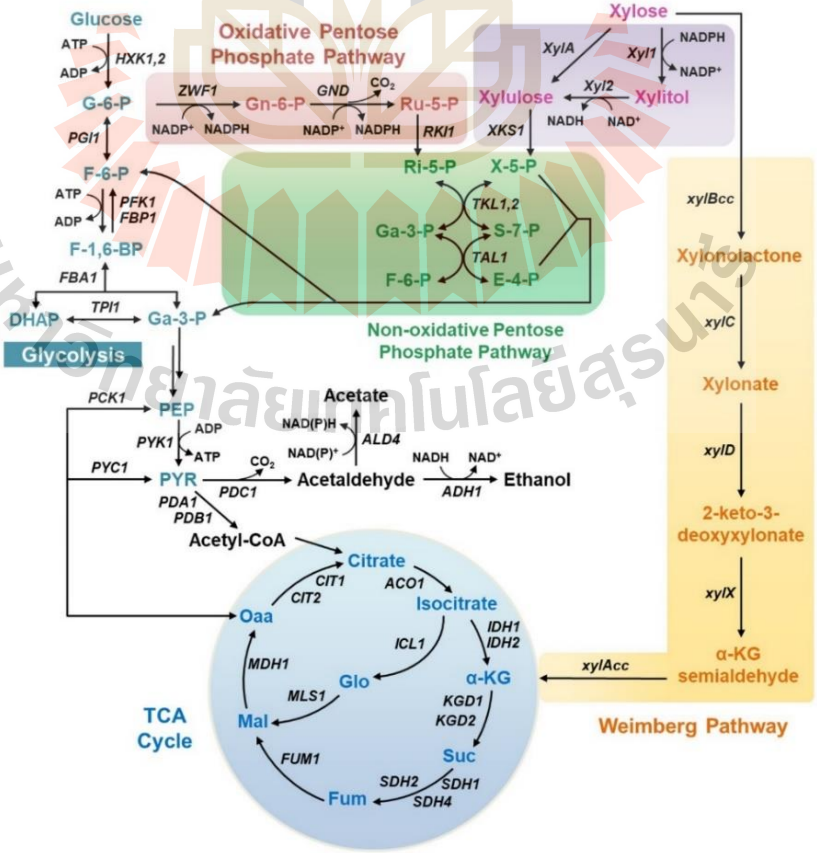

Figure 2.4 The yeast's xylose metabolism metabolic pathways (Zha et al., 2021).

2.1.2.4 Fourth generation (Photo-autotrophic microbes)

Photoautotrophs are organisms that use photons to obtain energy and use carbon dioxide to generate complex organic compounds (like carbohydrates). They are a more desirable host for biochemical reduction than cell-free and heterotrophic systems because of their capacity for photosynthesis and their reducing abilities. The ability of cyanobacteria to survive in adverse habitat conditions, the oldest class of photosynthetic organisms on Earth, aroused the researcher's interest in using them as a suitable host. But cyanobacteria either lack a natural system for transporting and reducing xylose or have a suppressed version of it (Ahuja et al., 2020).

2.2 Lignocellulosic biomass

The three polymers cellulose, hemicellulose, and lignin make up most of the lignocellulosic biomass, with tiny amounts of acetyl groups, minerals, and phenolic substituents also present (Figure 2.5). These polymers are arranged into complicated non-uniform three-dimensional structures to variable degrees and with varying relative compositions depending on the type of lignocellulosic biomass. The crystallinity of cellulose, the hydrophobicity of lignin, and the encapsulation of cellulose by the lignin-hemicellulose matrix all contribute to lignocellulose's robustness or recalcitrance (Isikgor & Becer, 2015).

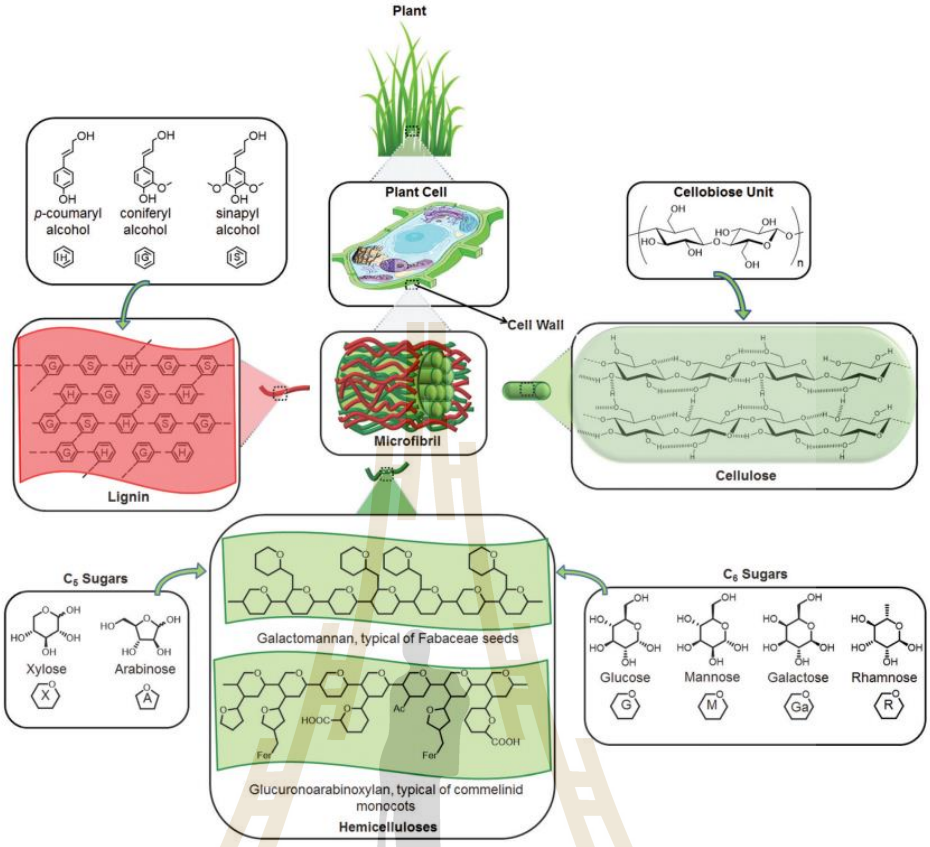

Figure 2.5 The principal elements and composition of lignocellulose. The letters "Gl" and "Fer" stand for glucuronic acid and ferulic acid, respectively, esterifications that are typical of xylans in commelinid monocots (Isikgor & Becer, 2015).

Cellulose makes up a large portion of lignocellulosic biomass. The repeating unit of the cellulose chain is the disaccharide cellobiose, as opposed to glucose in other glucan polymers. Its vast intramolecular and intermolecular hydrogen bonding networks closely connect the glucose units together to form its structure (Figure 2.5). The production of fuels and useful compounds from cellulose, which makes up nearly half of the organic carbon in the biosphere, is of utmost importance. Second in terms of polymer abundance is hemicellulose. Hemicellulose, in contrast to cellulose, is made up of a variety of heteropolymers, such as xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan (Figure 2.5). Hardwood hemicelluloses are primarily composed of xylans, whereas softwood hemicelluloses are primarily composed of glucomannan. Different pentose and hexose carbon monosaccharide units, including pentoses (xylose, arabinose), hexoses (mannose,

glucose, galactose), and acetylated sugars, make up the heteropolymers of hemicellulose. By joining cellulose fibers into microfibrils and cross-linking with lignin, hemicelluloses produce a complex network of linkages that are embedded in the plant cell walls to provide structural strength (Figure 2.5). Finally, lignin is a phenylpropanoid-based three-dimensional polymer. It serves as a cellular glue that gives plant tissue and individual fibers compressive strength, rigidity to the cell wall, and resistance to diseases and insects. The structure of lignin is produced by the oxidative coupling of three distinct phenylpropane building units, monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and just alcohol. As shown in Figure 2.5, the matching *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the phenylpropanoid monomer in the lignin polymer are each recognized. The distribution of lignin, cellulose, and hemicellulose in cell walls is not constant. Species, tissues, and plant cell wall maturity all affect how these components of the plant cell wall are arranged and how much of each there is. Typically, lignocellulosic biomass has 35 to 50% cellulose, 20 to 35% hemicellulose, and 10 to 25% lignin. The remaining portion consists of proteins, oils, and ash shown in Table 2.1 (Chen et al., 2022).

Table 2.1 Different lignocellulosic biomass and their chemical composition (Sitepu et al., 2014).

Lignocellulosic biomass		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hard wood	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Soft wood	Spruce	45.5	22.9	27.9
	Pine	42.0-50.0	24.0-27.0	20.0
Agricultural waste	Sugarcane bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Rice Straw	29.2-34.7	23.0-25.9	17.0-19.0
	Corn cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Coconut husk	34.0	21.0	27.0
	Mango peel	9.19	14.51	4.25
	Wheat straw	35.0-39.0	23.0-30.0	12.0-16.0
Grasses	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0

2.2.1 Green coconut husk

The fibrous outer layer of the coconut fruit, or the "husk," is a by-product of the extraction of copra and is typically regarded as waste. Coconut husk is a low-price, environmentally friendly, and renewable waste biomass. It has a high carbon concentration, low impurity level, and a wide range of uses in carbon-containing materials (Tian et al., 2022). The chemical compounds of coconut husk are shown in Table 2.2.

Table 2.2 Chemical compounds of coconut husk.

Compounds	Fraction percentage (%)
Ash, %	5.96
concentrate of protein (CP), %	3.82
Ether extract (EE), %	1.15
Coconut fiber (CF), %	47.27
Neutral detergent fiber (NDF), %	63.63
Acid detergent fiber (ADF), %	49.56
ADL, %	15.84

2.2.2 Sugarcane bagasse

About half to one third of plant tissues are made of cellulose, the primary component of all plant components, which is constantly regenerated by photosynthesis. Sugarcane bagasse, also known as "bagasse," is one of the most significant cellulosic agro-industrial by-products. It is an Abreus waste made up of cane stalks that are left over after the sugarcane is crushed and its juice is extracted. It is a lignocellulosic residue (by-product) of the sugar industry that is almost entirely utilized by the sugar factories itself as boiler fuel. More effectively using agro-industrial leftovers, such as sugarcane bagasse, has become more popular in recent years. It has been claimed that sugarcane bagasse is used as a raw material in a few processes and products. These include the creation of electricity, the manufacture of pulp and paper, and items made by fermentation. The production of enzymes and protein-enriched

bovine feed has been one of the primary uses of bagasse. The creation of various methods to manufacture protein-enriched cow feed is the result of a growing understanding of the benefit of using renewable resources, such as bagasse, for value addition. The simultaneous isolation and commercialization of cellulases enzymes have helped the economy recover considerably, even though such processes in submerged fermentation are adversely harmed by the high cost of product isolation (and low value of the product). Cellulases are also being utilized more and more to extract fruit juices, starch, and oil from woody materials, even though enzymatic saccharification of cellulose has been shown to be unprofitable. This approach is suitable for protein enrichment and cellulases generation from bagasse because these enzymes may be readily retrieved from fermented materials in solid-state fermentation of bagasse. The economic utilization of bagasse-based processes is still restricted, despite these advancements (Ajala et al., 2021).

2.3 Pretreatment of lignocellulosic material

Fractionating lignocellulose into its three principal components such as cellulose, hemicelluloses, and lignin. They are one of the most crucial objectives of lignocellulosic biomass refinement. Pyrolysis and other single-step therapy techniques are ineffective. The lignocellulosic biomass is deconstructed since these procedures typically rely on high temperatures, even though they result in cheaper prices. The created bio-oil is made up of a complex mixture of hundreds of different components, making it extremely difficult and inconvenient to extract the desired chemicals and fuels in a single process. Additional expenses and a variety of pretreatment techniques are needed for downstream separations to be effective. By altering the supramolecular structure of the cellulose-hemicellulose-lignin matrix, the pretreatment procedures change the lignocellulosic materials' inherent binding properties. To maximize cellulose and hemicellulose accessibility and biodegradability for enzymatic or chemical action, pretreatment of lignocellulosic biomass is therefore crucial before using other treatment methods (Sitepu et al., 2014). Pretreatment techniques fall under a variety of categories, including mechanical, chemical, physicochemical, biological, and various combinations of these. It has been observed that a variety of pretreatment techniques can hydrolyze, solubilize, and separate the components of cellulose, hemicellulose, and lignin. Some of them are wet oxidation, ozonolysis, dilute- and concentrated-acid hydrolyses, biological pretreatments, milling, irradiation, microwave, steam explosion, ammonia fiber explosion (AFEX), supercritical CO_2 and its explosion, SO_2, alkaline hydrolysis, liquid hot-water pretreatment, and organic solvent processes. These techniques all aim to shrink the biomass and expose its physical structure. According to reports, each of these techniques has benefits and drawbacks.

Pretreatment of lignocellulosic biomass holds significant promise for increasing productivity and reducing costs through research and development. It will be significantly less expensive to use lignocellulose for practical applications if different biomass pretreatment techniques are combined with other procedures such enzymatic saccharification, detoxification, fermentation of the hydrolysates, and recovery of products. As a result, advancements in pretreatment technologies, microorganisms that produce cellulolytic enzymes, the best possible exploitation of the components of biomass, and process integration are all likely to be key factors in the future success of lignocellulosic conversion on a commercial scale (Kaur et al., 2022).

2.4 Detoxification of lignocellulosic hydrolysates

The hemicellulose hydrolysate produced by the pretreatment process contains a variety of undesirable byproducts, including colorants, inorganic salt, acetic acid, furfural, and hydroxy-methyl-furfural (HMF). These substances need to be eliminated before or after fermentation since they could hinder it and alter the yield of the final product. To do this, the fermentation broth is combined with activated charcoal and then filtered. The method works well for clarifying broth and removing colorants. Ion exchange resins, including cation-exchange and anion exchange resins sequentially, are another option for removing ionic charged pollutants besides activated charcoal. Cation-exchange resins are used for desalination and the removal of organic chemicals that have positive charges as opposed to anion exchange resins, which are employed for anionic colored compounds. Most pollutants may be removed effectively and affordably using an ion exchange resin and activated charcoal treatment (Mun et al., 2016).

2.5 Fermentation

Bacteria, yeasts, and certain fungi among other microbes engage in the metabolic process of fermentation. Organic substances like sugars and carbohydrates are converted during fermentation into less complex substances like alcohol, organic acids, and gases. Without the need for oxygen, the process frequently produces ATP (adenosine triphosphate), which is a kind of energy. For thousands of years, people have utilized fermentation to create foods and drinks including bread, beer, wine, cheese, and yogurt. Pharmaceuticals, enzymes, and biofuels are also made using it. The fermentation process can be impacted by a wide range of factors, including temperature, pH, and the presence or lack of specific nutrients. A variety of substrates can be fermented by different microbes, and these end products can then be used in a variety of industrial processes (Ezemba et al., 2022).

2.5.1 Batch fermentation

A significant volume of nutrient medium is infected in a closed system known as batch fermentation to move on with the harvest and recovery of the product. As the vessel is cleaned and stabilized for the succeeding batches, the batch fermentation comes to an end. All the nutrients are initially introduced to the vessel and infected in this tight arrangement. As part of subsequent treatments, stirrers are used to ensure proper aeration and an acid or alkali is added to adjust pH. Antifoam ingredients like soybean or palm oil are added to aerated stems. Microorganisms that are growing on a large scale may cause the system to become hot. By establishing a water circulation system around the vessel for heat exchange, temperature control is maintained. The growth of microorganisms in batch fermentation follows the typical growth curve, with a lag phase, followed by a log phase, and finally reaching the stationary phase due to nutritional limitation and other variables. When using complex nutrition solutions, a growth curve might be seen. Due to the preferential use of one of the substrates, two lag phases typically occur, followed by a second log phase. The breakdown of one substrate is inhibited by the presence of another substrate. In conclusion, before products are collected, microorganisms go through all the stages of growth (Lag phase, transient acceleration phase, exponential phase, deceleration, stationary phase) (Ezemba et al., 2022).

2.5.2 Fed-batch fermentation

In a fed-batch process, one or more nutrients are delivered to the bioreactor but there is no outflow while the process is taking place. By changing the feed rate during the run-in response to the feedback of control parameters like dissolved oxygen (DO), pH, or respiratory quotient (RQ) (Minihane & Brown, 1986). It is possible to externally modify the concentration of one or more nutrients in the medium. By adding nutrients at different stages of fermentation, the growth of the microorganisms can be sustained for longer periods of time, resulting in higher cell density and ultimately higher product yield. This is one of the benefits of fed-batch fermentation over batch fermentation. Nutrients can be provided to fed-batch fermentation in a regulated way to maximize the microorganisms' access to them. This increases the fermentation process' efficiency and maintains the rate of microbial development. Fed-batch fermentation, in general, enables greater environmental control, which can lower the accumulation of harmful metabolites that might impede the growth of the microorganisms and lower the yield of the product. Additionally, fed-batch fermentation is a versatile technique that can be adjusted to various microbe varieties and fermentation environments. In addition, it can be used to make a variety

of goods, including medicines, biofuels, and enzymes. The batch-to-batch unpredictability that is typical of batch fermentation can also be lessened using fed-batch fermentation. The fermentation environment can be managed, and nutrients can be added in a regulated way to increase the product's quality and yield (Minihane & Brown, 1986).

2.6 Response surface methodology

The response surface methodology (RSM) is a group of statistical and mathematical methods for developing empirical models. A response (output variable) that is impacted by several independent variables (input variables) is to be optimized through proper experiment design. An experiment is a collection of tests, or runs, in which the input variables are altered to determine the causes of variations in the output response (Kavya & Nadumane, 2023).

RSM was initially created to model experimental reactions before moving on to model numerical experiments (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. The distinction is in the type of mistake that the response produces. For instance, measurement errors can cause inaccuracy in physical experiments, whereas round-off errors, poor iterative process convergence, or the discrete representation of continuous physical events can cause numerical noise in computer experiments (Giunta et al., 1996; van Campen et al., 1990, Toropov et al., 1996). It is presumed that the errors in RSM are random.

RSM is being applied to design optimization with the goal of lowering the expense of costly analysis techniques (such the finite element method or CFD analysis) and the numerical noise they produce. Smooth functions that limit the effects of noise and enable the employment of derivative-based techniques can be used to approximate the problem as it is described, improving the convergence of the optimization process (Sarabia & Ortiz, 2009).

2.6.1 Design of experiments

Design of experiments, or DOE as it is commonly referred to, is a crucial component of RSM. These methods can be applied to numerical experiments even though they were initially developed for the model fitting of physical experiments. The identification of the points at which the response should be examined is the DOE's goal. The mathematical model of the process relates to most of the criteria for the best experiment design. Since these mathematical models are frequently polynomials with unknowable structures, specific tests are developed for each distinct problem. The choice of experiment design can have a significant impact on the response surface construction cost as well as the approximation's accuracy (Sarabia & Ortiz, 2009).

In a traditional DOE, screening experiments are performed in the early stages of the process, when it is likely that many of the design variables initially considered have little or no effect on the response. The purpose is to identify the design variables that have large effects for further investigation. A detailed description of the design of experiments theory can be found in Box and Draper (1987), Myers and Montgomery (1995) and Montgomery (1997), among many others. Schoofs (1987) has reviewed the application of experimental design to structural optimization, Unal et al. (1996) discussed the use of several designs for response surface methodology and multidisciplinary design optimization and Simpson et al. (1997) presented a complete review of the use of statistics in design (Sarabia & Ortiz, 2009).

2.6.2 Box-Behnken design

When designing experiments, the response surface methodology (RSM) technique known as the Box-Behnken design is often used to explore and optimize the correlations between various input variables and one or more response variables. It is a sort of experimental design that entails carrying out tests with various degrees of input variables to identify the ideal circumstances for a process. The DOE method comparison is depicted in Figure 2.6 (Box & Draper, 1959).

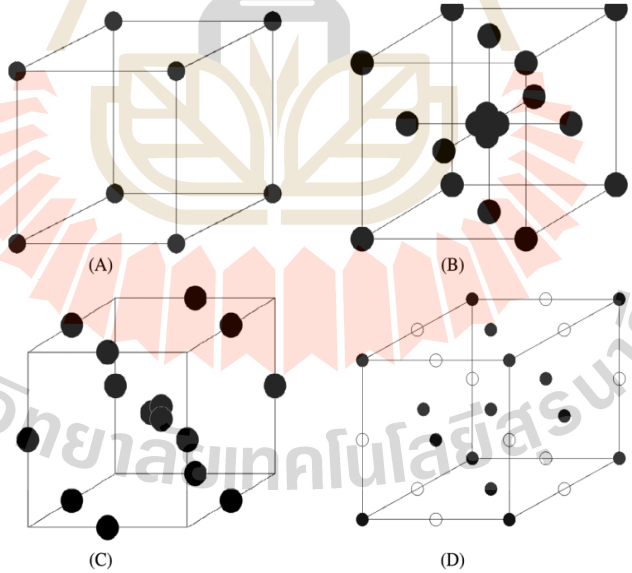

Figure 2.6 Three experimental designs are shown schematically: (A) two-level full factorial design; (B) face-centered central composite design; (C) Box-Behnken design; and (D) three-level full factorial design (Box & Draper, 1959).

The experiments of a Box-Behnken design are run at the midpoint of each cube face, with extra tests run at the center point, and the input variables are adjusted

within three levels. A second-order polynomial model that can be used to forecast the response values at any location along the design space can be created thanks to the design. The Box-Behnken design has the advantage of requiring fewer experiments than a full factorial design, which is one of its main advantages. In contrast to a full factorial design, it can be utilized to construct a response surface model with fewer experiments. Because of the design, it is possible to analyze the data using statistical methods like regression analysis and analysis of variance (ANOVA) to determine how the input variables affect the outcome variables. Additionally, the design enables the selection of the ideal conditions for the process based on the response surface model (Box & Draper, 1959).

In response surface technique, the Box-Behnken design kind of experimental design is employed to optimize and investigate the correlations between numerous input variables and one or more response variables. With fewer experiments required, it enables the creation of a response surface model that may be used to improve the process and identify its ideal circumstances (Box & Draper, 1959).

CHAPTER 3
MATERIALS AND METHODS

3.1 Sugarcane bagasse and green coconut husk

Sugarcane bagasse and green coconut husk were obtained from Suranaree University of Technology' Farm and Ratchaburi's coconut company respectively. The samples were dried in a hot air oven at 60 °C for 2 days. After that extracted of lignin and tannin with 95% ethanol and dried in a hot air oven at 70 °C for 24 h. The dried sugarcane bagasse and dried green coconut husk were cut into small pieces and ground into 500 µm by the grinder machine. The ground SCB and ground GCH were packed in plastic bag and stored in a dry place for further use.

3.2 Collection and isolation of xylitol producing microorganisms

Soil samples were collected in Thailand from various sites (Saraburi: 14.30 °N latitude and 100.55 °E longitude, Nakhon Ratchasima: 14.59 °N latitude 102.12 °E longitude, Ratchaburi: 13.30 °N latitude and 99.54 °E longitude, and Kanchanaburi: 14.02 °N latitude and 99.31 °E longitude) for example corn field, sugarcane field, rice field. Samples of corn, rice, sugarcane bagasse, green coconut husk, and sugarcane were also collected during the year of 2022 for isolation of xylitol producing microorganism. The samples were collected using the sterilized bottle and stored at 4 °C until use. The collected samples (1% w/v) were added in enrichment medium containing xylose 10 g/L and peptone 3 g/L (XP broth) pH 5.5 incubated at 30 °C for 48 h to promote the growth of xylitol producing microbial stains (Lugani & Sooch, 2020). Xylose was added to the enrichment medium to promote the growth of xylose utilizing microorganisms because it is required in xylitol producing pathway. Thereafter, the samples were streaked on an agar plate containing xylose 10 g/L, peptone 3 g/L, and agar 20 g/L (XP agar). These plates were incubated at 30 °C for 48 h. Isolated colonies were picked up and subculture on a new plate 3-4 times. The isolated pure culture of yeast and bacterial were maintained on nutrient agar slants containing

peptone (5.0 g/L), NaCl (5.0 g/L), beef extract (1.5 g/L), yeast extract (1.5 g/L) and agar (20 g/L) with pH 6.0. The microorganism was stored at 4 ± 1 °C until use.

The isolated microorganisms were picked up to XP broth and incubated in shaker incubator at 30 °C until became turbid due to microbial growth. After that, take 500 µl of culture in sterilized microtube with 500 µl of 40% glycerol solutions, mix thoroughly and keep in the deep freezer (-80 °C).

3.3 Screening of xylitol production from microorganisms

The starter medium for isolated microorganisms was prepared using YPD medium containing peptone 20 g/L, yeast extract 10 g/L, and dextrose 20 g/L with pH 5.5 and incubated at 30 °C for 24 h in incubator shaker at 150 rpm. Then, add 10% v/v of the starter culture were aseptically transferred to the fermentation medium containing xylose 20 g/L and peptone 6 g/L with pH 5.5 incubated at 30 °C for 24 h in incubator shaker at 150 rpm. The cell biomass was harvested by centrifugation at 5000×g for 10 min at 4 ± 1 °C (260 D, Denville Scientific, USA).

3.4 Analysis of xylitol production

After cultivation, the supernatant was analyzed. Isolated microorganisms were tested for xylitol production by qualitative and quantitative methods. The qualitative analysis using thin layer chromatography method (TLC). Solvent system of TLC plate containing water: ethyl acetate: propanol (1:2:7) and using *p*-aminobenzoic acid and sodium periodate for spraying (Lugani & Sooch, 2020). The quantitative analysis using high performance liquid chromatography (Chromaster, Hitachi, Japan) by aminex hpx-87h column. The mobile phase was used 8 mM H_2SO_4. The flow rate was maintained at 0.600 mL/min and column temperature was maintained at 45 °C (Vaz de Arruda et al., 2017).

3.5 Identification of isolated microbial

3.5.1 Morphological characterization

Colonies of the isolated microbial such as colony color, surface, form, appearance, elevation, and margin were observed after incubated in plates for 48 h at 30 °C.

3.5.2 Genome characterization

For genomic DNA extraction, the isolated strains were weakened by lithium acetate (LiOAc) and SDS solution. After 24 h of cultivation in YPD medium, 200 µL of the broth was centrifuged at 15,000 ×g for 5 min. The cell pellet was re-suspended in 100 µL of 200 mM LiOAc with 1% SDS solution, centrifuged at 15,000 ×g for 3 min and

incubated at 70 °C for 15 min. After incubation, 300 µL of 96% ethanol was added for DNA precipitation, mixed using brief vortexing, and centrifuged at 15,000 ×g for 3 min. After that, it was re-suspended in 500 µL of 70% ethanol, centrifuge at 15,000 g for 3 min, and evaporated at room temperature for 5 min. Precipitated DNA was dissolved in 100 µL of TE buffer and centrifuge at 15,000 ×g for 15 s. And 1 µL of the supernatant was used for PCR reaction (Looke et al., 2011).

After genomic DNA extraction, 1 µL of the supernatant was amplified using the internal transcribed spacer 5.8S (ITS-5.8S) and domains 1 and 2 of the 26S rDNA regions of the isolated microorganism (Gosalawit et al., 2020). The primers ITS1_F, ITS4_R, NL1_F and NL4_R were used for PCR reaction. The PCR were preformed in 25 µL reaction volume containing 1X PCR buffer (20 mM Tris HCl (pH 8.4) and 50 mM KCl), 2 µL (each) 2 mM deoxyribonucleoside triphosphate, 1 µL of 10 mM of each primer, 0.5 µL *Taq* polymerase, 17 µL of PCR Grade Water, and 1 µL of DNA template. Reaction mixtures were subjected to initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, and polymerization for 1 min at 72 °C in the reaction mixtures. The last extension phase was carried out at 72 °C for 10 min. Sterilized deionizer water was used as a negative control. PCR products were analyzed by electrophoresis on 2% agarose gel with 1X TAE buffer (40 mM tris aminomethane base, 20 mM acetic acid, and 1 mM EDTA). Ethidium bromide staining was followed by UV irradiation to reveal the resultant bands. The approximate size of amplicons was calculated using standard molecular weight markers (100–2000 bp, Promega, Madison, USA). Molecular identification of the new isolated was based on next generation sequence analysis of the internal transcribed spacer by Macrogen, Inc. (South Korea). The obtained sequence was BLAST against NCBI database (Gosalawit et al., 2020).

Table 3.1 Primers used in this study (Gosalawit et al., 2020).

Primer	Sequence	Tm (°C)
ITS1_F	5'-TCCGTAGGTGAACCTGCGG-3'	68.40
ITS1_R	5' TCCTCCGCTTATTGATATGC-3'	61.50
NL1_F	5'-GCATATCAATAAGCGGAGGAAAAG-3'	65.34
NL4_R	5'-GGTCCGTGTTTCAAGACGG-3'	65.53

3.6 Pretreatment of sugarcane bagasse and green coconut husk using dilute acid

Different physiological parameters such as temperature, time, solid-liquid ratio, and concentration of acid affect the concentration of monomeric sugar such as xylose, arabinose, and glucose after hydrolysate. Those parameters are significant for xylitol fermentation. In this study optimization of parameters for hydrolysate of GCH and SCB using dilute sulfuric acid used experimental design with DOE (Vardhan et al., 2022). The amount of sugar (glucose and xylose) obtained in lignocellulosic hydrolysate was analyzed by high performance liquid chromatography (HPLC; Chromaster, Hitachi, Japan) using aminex hpx-87h column. The mobile phase was 8 mM H_2SO_4. The flow rate was maintained at 0.600 mL/min and column temperature was maintained at 45 °C. Experimental design was used DOE with Design Expert 13 (Vardhan et al., 2022).

3.6.1 Experiment design of pretreatment lignocellulosic material

The Response Surface Methodology (RSM) was used for the modelling and optimization process (Design Export 13) with input parameters of time (10-30 min), temperature (110-130 °C), concentration of acid (0-15% (v/v)), and solid loading (5-20% (w/v)) (Table 1). The Box-Behnken design was generated using Design Export 13 software. The software-generated study design was used in triplicate, and predictions and actual results regarding the xylose were examined. Three copies of each of the twenty-seven different responses found in the design expert matrix were examined, and their means were taken into account (Box & Draper, 1959).

Table 3.2 Evaluated factors, factor notation, and their levels in Box-Behnken for SCB and GCH hydrolysate

Independent variables	Code	Factor levels		
		-1	0	1
Time (min)	A (X_1)	10	20	30
Temperature (°C)	A (X_2)	110	120	130
Acid (%)	A (X_3)	0	7.5	15
Solid (%)	A (X_4)	5	12.5	20

In the method, the following equation was used to model the relationship between the response variable (Y_i) and the predictor variables:

$$Y_i = \beta_0 + \Sigma\, \beta_i x_j + \Sigma\, \beta_{ii} x_j^2 + \Sigma\, \beta_{ij} x_i x_j \qquad (3.1)$$

In this equation, i and j represent the linear and quadratic coefficients, respectively. β_0 represents the intercept coefficient, β_i represents the linear effect, β_{ii} represents the quadratic effect, and β_{ij} represents the interaction effect. The response variable Y represents the concentration of xylose. To assess the fitness of the β model, analysis of variance (ANOVA) was performed using Design Expert software version 13

(Kavya & Nadumane, 2023). The ANOVA analysis helps evaluate the statistical significance of the model and the individual coefficients (β) in predicting the response variable.

3.6.2 Detoxification of lignocellulosic hydrolysate

After hydrolysate by the optimum condition with high xylose rich, GCH hydrolysate was detoxified by activated carbon (Loba Chemie™, India). The detoxification was performed in Erlenmeyer flask at 25 °C by an incubator shaker (200 rpm rotation) for 1 h with activated carbon loading 3% (w/v) (Dasgupta et al., 2022).

3.7 Effect of growth conditions on xylitol production

The most important factors that affect growth and xylitol production by microorganism strains were studied. Different concentrations of xylose, yeast extract, potassium dihydrogen phosphate (KH_2PO_4) and ammonium sulfate $(NH_4)_2SO_4$ (Vaz de Arruda et al., 2017) were evaluated on the growth and production of xylitol.

Meyerozyma guilliermondii C511C was used in this experiment. The inoculum was cultured in a 125 mL Erlenmeyer flask with 25 mL of YPD broth medium at 30 °C on incubator shaker. After 24 h of cell growth (OD_{600} = 0.6), transfer 10% (w/v) of starter to 250 mL Erlenmeyer flask with 50 mL of medium containing xylose (10-30 g/L), yeast extract (1-5 g/L), KH_2PO_4 (0-3 g/L), and $(NH_4)_2SO_4$ (0-2 g/L) (Table 2). The second carbon source; glucose was maintained at 5 g/L and 0.1 g/L of magnesium sulfate ($MgSO_4$) was added. The inoculum loading was fixed at 10% (w/v) since it is the standard acceptable quantity usually implemented for xylose fermentation processes (Kaur et al., 2022). The temperature, time, pH, and speed of incubator shaker were fixed at 30 °C, 72 h, 5.5, and 150 rpm. respectively. The Box-Behnken design was generated using Design Export 13 software. The study design obtained by the software was adopted in triplicate and the predicted and obtained responses regarding the xylitol production were analyzed. The twenty-seven different responses obtained in the design expert matrix were studied in triplicate, and their means were considered. All cell growth measurements were analyzed by spectrophotometer (Gosalawit et al., 2021) at OD_{600}, the biomass was suspended in 1 mL of deionizes water, concentration of xylitol, xylose and glucose were detected by HPLC (Vaz de Arruda et al., 2017). The ideal conditions for fermentation in a 500 mL Erlenmeyer flask were identified initially, and then the optimum conditions were maintained and scaled up to a 5-L bioreactor.

Table 3.3 Evaluated factors, factor notation, and their levels in Box-Behnken.

Independent variables	Code	Factor levels		
		-1	0	1
Xylose (g/L)	A (X_1)	10	20	30

Yeast extract (g/L)	A (X_2)	1	3	5
KH_2PO_4 (g/L)	A (X_3)	0	1.5	3
$(NH_4)_2SO_4$ (g/L)	A (X_4)	0	1	2

In the method, the following equation was used to model the relationship between the response variable (Y_i) and the predictor variables:

$$Y_i = \beta_0 + \Sigma\, \beta_i\, x_j + \Sigma\, \beta_{ii}\, x_j^2 + \Sigma\, \beta_{ij}\, x_i\, x_j \qquad (3.2)$$

In this equation, i and j represent the linear and quadratic coefficients, respectively. β_0 represents the intercept coefficient, β_i represents the linear effect, β_{ii} represents the quadratic effect, and β_{ij} represents the interaction effect. The response variable Y represents the concentration of xylose. To assess the fitness of the **β** model, analysis of variance (ANOVA) was performed using Design Expert software version 13 (Kavya & Nadumane, 2023). The ANOVA analysis helps evaluate the statistical significance of the model and the individual coefficients (**β**) in predicting the response variable.

3.8 Batch fermentation with 5-L bioreactor

The cultivation was developed in a 5-L bioreactor. with 3.5 L working volume. Microorganism cultivation was measured of cell growth rate by spectrophotometer at OD_{600} (Gosalawit et al., 2021), concentration of monomeric sugar and xylitol production was analyzed by HPLC (Hu et al., 2012). The inoculum was cultured in a 500 mL Erlenmeyer flask with a volume of 250 mL using YPD medium. After 24 h, the inoculum 10% (v/v) was transferred into the 5-L bioreactor. with xylose 30 g/L, glucose 5 g/L, yeast extract 3 g/L, ammonium sulfate 2 g/L, potassium dihydrogen phosphate 2 g/L, and magnesium sulfate 0.1 g/L. The Sartorius stedim, Biostat Bpus (5 L total volume, the working volume of 3.5 L) bench bioreactor was used for this process. During fermentation, the pH was automatically adjusted to 5.5. Other conditions of fermentation were temperature 30 °C, agitation 300 rpm, and aeration rate was maintained at 1 vvm. All experiments were terminated when xylitol concentration reached its maximum value and tended to decrease. The obtained results were compared by measuring HPLC, sugar consumption, xylitol yield (g of xylitol/g of xylose) (Wannawilai et al., 2017), and optical density. All experiments were performed by three replicates for each experiment.

3.9 Fed-batch fermentation with 5-L bioreactor

The Sartorius stedim, Biostat Bpus (5 L total volume, the working volume of 3.5 L) bench bioreactor was used for this process. The inoculum was cultured in a 500 mL Erlenmeyer flask with a volume of 250 mL using YPD medium. After 24 h, the inoculum

10% (v/v) was transferred into the 5-L bioreactor. with xylose 30 g/L, glucose 5 g/L, yeast extract 3 g/L, ammonium sulfate 2 g/L, potassium dihydrogen phosphate 2 g/L, and magnesium sulfate 0.1 g/L. After 18 h and 48 h of fermentation, add xylose 20 g/L to the bioreactor. During fermentation, the pH was automatically adjusted to 5.5. Other conditions of fermentation were temperature 30 °C, agitation 300 rpm, and aeration rate was maintained at 1 vvm. All experiments were terminated when xylitol concentration reached its maximum value and tended to decrease. The obtained results were compared by measuring HPLC, sugar consumption, xylitol yield (g of xylitol/g of xylose) (Wannawilai et al., 2017), and optical density at OD_{600}. All experiments were performed by three replicates for each experiment.

3.9.1 Fed-batch fermentation with 5-L bioreactor using GCH hydrolysate

The Sartorius stedim, Biostat Bpus (5 L total volume, the working volume of 3.5 L) bench bioreactor was used for this process. The inoculum with *M. guilliermondii* C511C was cultured in a 500 mL Erlenmeyer flask with a volume of 250 mL using YPD medium. After 24 h, the inoculum 10% (v/v) was transferred into the 5-L bioreactor. with xylose from GCH hydrolysate 30 g/L, glucose 5 g/L, yeast extract 3 g/L, ammonium sulfate 2 g/L, potassium dihydrogen phosphate 2 g/L, and magnesium sulfate 0.1 g/L. After 30 h of fermentation, add xylose 20 g/L to the bioreactor. During fermentation, the pH was automatically adjusted to 5.5. Other conditions of fermentation were temperature 30 °C, agitation 300 rpm, and aeration rate was maintained at 1 vvm. All experiments were terminated when xylitol concentration reached its maximum value and tended to decrease. The obtained results were compared by measuring HPLC, sugar consumption, xylitol yield (g of xylitol/g of xylose), and optical density at OD_{600}. All experiments were performed by three replicates for each experiment (Wannawilai et al., 2017).

3.9.2 Analysis method

3.9.2.1 Fermentation broth

The parameters in the fermentation broth that were being monitored over time were cell concentration, residual xylose, xylitol content, and yield xylitol production (Kumar et al., 2015).

3.9.2.2 Cells concentration

Deionized water was used to wash the cells twice. By measuring optical density (OD) at 600 nm, yeast growth was quantified (Wannawilai et al., 2017).

3.9.2.3 Monomeric sugar analysis

After fermentation, the supernatant was filtered through 0.22 μm and analyzed for monomeric sugar. The monomeric sugar; xylose, xylitol, and glucose were determined with HPLC (Chromaster, Hitachi, Japan) using aminex hpx-87h column. The mobile phase was used 8 mM H_2SO_4. The flow rate was maintained at 0.600 mL/min, column temperature was maintained at 45 °C, and retention time 20 min with 20 μL injection volume (Vaz de Arruda et al., 2017).

The concentration of xylose, xylitol, and glucose were calculated from the standard curve. A diagram of change in xylose, xylitol concentration over time were also generated (Kumar et al., 2015). The xylitol yield (g/g) (Kumar et al., 2015)

$$Y_x = \frac{\text{Xylitol produced (g)}}{\text{Xylose consumed (g)}} \quad (3.3)$$

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening and isolation of microbial for xylitol production

In the experiment, an enrichment media containing xylose was used to promote the growth of microorganisms capable of utilizing xylose. Xylose is essential for the metabolic activity of the xylose reductase enzyme, which is involved in the conversion of xylose to xylitol. A total of 36 strains were isolated from various sources in Thailand, and their ability to utilize xylose and potentially produce xylitol was assessed. The screening of the isolated strains was performed using TLC (Thin-Layer Chromatography) method and HPLC (High-performance liquid chromatography) analysis. The quantitative estimation of xylitol production from the isolated microbial strains was carried out following the method described by Vas de Arruda *et al*. The chromatogram results of the best 19 isolated strains, along with their corresponding xylitol concentrations, are presented in Table 4.1. Among all the isolated strains, the highest xylitol yield (0.623 g/g) was observed in the C511C strain. The chromatogram representing the xylitol production of this strain is illustrated in Figure 4.1. These findings highlight the potential of the C511C strain for efficient xylitol production and suggest its suitability for further investigation and application in the field.

In generally there are many different types of samples that contain xylitol-producing microorganisms, such as *Psudomonas putida* BSX-46 from bagasse (Lugani & Sooch, 2020), *Debaryomyces hansenii* from overripe grapes (Prakash et al., 2011), *C. sojae* JCM 1644 from rotten fruit juices (Pant et al., 2022), *C. tropicalis* from Brazilian sugarcane crops (Bevilaqua et al., 2023), and *Meyerozyma caribbica* 5XY2 from an alcohol fermentation starter (Sukpipat et al., 2017). Xylitol-producing microorganisms were screened and isolated in earlier investigations, and among the isolated strains. In previous studies, the screening and isolation of xylitol-producing microorganisms were conducted, and among the isolated strains, yeast was found to be the preferred choice for xylitol production. Yeast strains offer several advantages over bacteria in terms of xylitol production. They have the potential to achieve higher yields of xylitol compared

to bacteria, as certain yeast species exhibit higher specific activity of the xylose reductase enzyme and produce xylitol at faster rates. This increased efficiency can lead to greater overall xylitol production and improved productivity. Additionally, yeasts generally possess higher tolerance to inhibitory substances commonly found in lignocellulosic hydrolysates, which are frequently used as feedstocks for xylitol production. These inhibitory substances, including furfural and acetic acid, can have detrimental effects on microbial growth and xylitol production. However, yeast strains have demonstrated robustness and the ability to withstand such inhibitory conditions, making them more suitable for xylitol production from complex lignocellulosic materials. Considering these factors, yeast emerges as a favorable choice for xylitol production due to its ability to achieve higher yields, faster production rates, and greater tolerance to inhibitory compounds commonly found in lignocellulosic feedstocks. These advantages position yeast as a promising candidate for efficient and sustainable xylitol production in various industrial applications (Dasgupta et al., 2017).

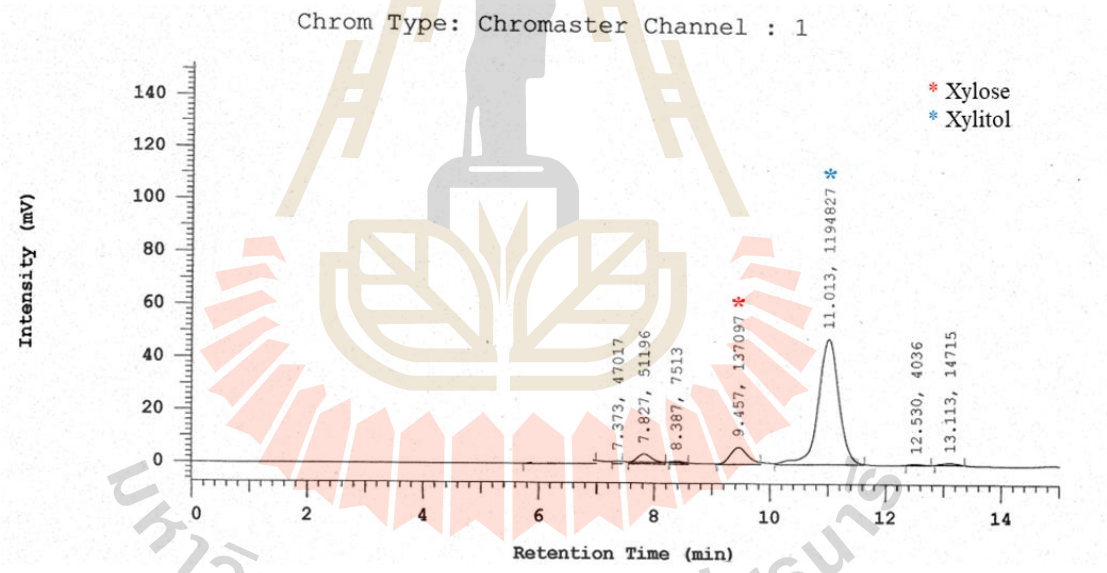

Figure 4.1 HPLC chromatogram of media of microbial C511C at 66 h from HPLC.

Table 4.1 Xylitol production of top 19 isolated with yield.

Microbial isolations	Field and location	After 66 h of fermentation (g/L)	Yield (g/g)

		Xylose	Xylitol	
C511C	Coconut husk, Ratchaburi	5.240	15.550	0.623
542C	Sugarcane bagasse, Saraburi	2.390	16.460	0.591
C511D	Coconut husk, Ratchaburi	6.774	13.744	0.587
C512F	Coconut husk, Ratchaburi	4.740	14.650	0.575
542D	Sugarcane bagasse, Saraburi	0.800	16.680	0.567
562B	Corn seed, Saraburi	0.000	17.050	0.564
592A	Soil from sugarcane field, Saraburi	0.000	17.060	0.564
592C	Soil from sugarcane field, Saraburi	6.150	13.530	0.563
C512B	Coconut husk, Ratchaburi	6.040	13.550	0.561
C512G	Coconut husk, Ratchaburi	0.000	16.830	0.556
591A	Soil from sugarcane field, Saraburi	5.640	13.670	0.556
C511B	Coconut husk, Ratchaburi	0.000	16.690	0.552
511D	Coconut husk, Ratchaburi	5.400	13.660	0.551
571B	Corncob, Saraburi	0.000	16.370	0.541
5101A	Cornhusk, Saraburi	8.620	11.530	0.535
C512C	Coconut husk, Ratchaburi	11.520	9.780	0.525
581A	Rice seed, Saraburi	1.400	15.020	0.521
592B	Soil from sugarcane field, Saraburi	3.120	13.780	0.508
561A	Soil from corn field, Saraburi	0.000	15.050	0.497

The initial concentration of xylose was 30 g/L and xylitol yield were calculated by this equation;

$$Y_X = \frac{\text{Xylitol produced (g)}}{\text{Xylose consumed (g)}} \quad (4.1)$$

4.2 Identification of isolated microbial

The morphological and genotypic characterization of the 10 isolated strains were conducted, focusing on the ITS rDNA and D1/D2 domain of the 26S rDNA. The results of ITS and D1/D2 amplification are shown in Figure 4.2 and Table 4.2, providing valuable insights into the relatedness of isolated microbials. Based on the ITS rDNA and D1/D2 domain analysis, six of the isolated microbials, namely 5101A, 542C, 562B, 591A, 571B, and 561A, exhibited a close relatedness to *Candida tropicalis*, with a pairwise similarity of 100%. This finding suggests that these isolates likely belong to

the *C. tropicalis* species. The high similarity in their ITS rDNA and D1/D2 domain sequences further supports their close genetic relationship.

Similarly, two of the isolated microbials, 581A and C512G, demonstrated a close relatedness to *Meyerozyma carpophila*, with a pairwise similarity of 100%. This indicates that these isolates are likely members of the *M. carpophila* species. The 100% similarity in their ITS rDNA and D1/D2 domain sequences strengthens the evidence for their close genetic affiliation. Lastly, the remaining two isolated microbials, C511C and C511D, exhibited a close relatedness to *Meyerozyma guilliermondii*, with a pairwise similarity of 100%. These isolates are likely representatives of the *M. guilliermondii*, as supported by their identical ITS rDNA and D1/D2 domain sequences. The high pairwise similarities of 100% identified within each group of microorganisms suggest a significant degree of genetic similarity between the individuals of the same species. This result shows that the individual species groupings have a close evolutionary connection and genetic homogeneity (Gosalawit et al., 2021).

The previous study report about the xylitol producing microbial was found in *C. tropicalis* CCTCC M2012462 about 0.7 g/g of yield with 0.46 g/L/h of productivity with corncob hydrolysate medium (Ping et al., 2013).

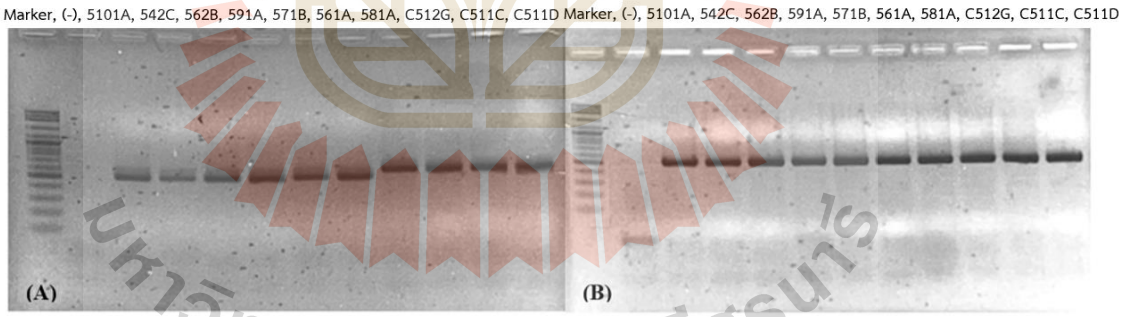

Figure 4.2 Gel electrophoresis of PCR products (A) ITS rDNA (B) D1/D2 domain of the 26S rDNA.

The SEM analysis of *C. tropicalis*, *M. carpophila*, and *M. guilliermondii* after 48 h cultivation in YPD broth is shown in Figure 4.3.

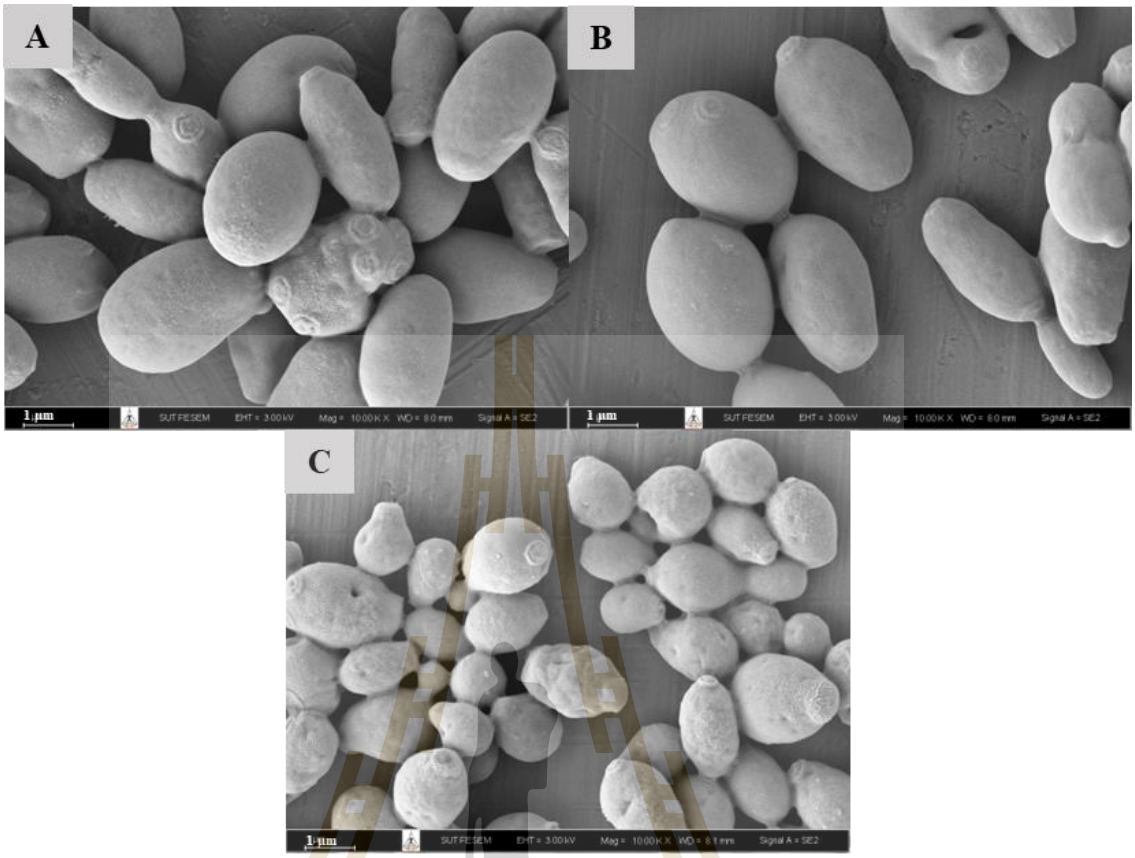

Figure 4.3 SEM images of (A) C. tropicalis 542C, (B) *M. carpophila* C512G, and (C) *M. guilliermondii* C511C.

The choice of microorganisms for xylitol production is an important consideration, not only in terms of their efficiency in converting sugars to xylitol but also in terms of their safety and potential for pathogenicity. *Candida* sp. have been widely studied and have demonstrated high yields of xylitol production using various carbon sources, including pure sugars and hemicellulose hydrolysate. However, it is important to note that some *Candida* sp., including *C. tropicalis*, can be opportunistic pathogens and pose potential risks in certain settings.

In contrast, *M. guilliermondii* is a non-pathogenic yeast that offers several advantages for xylitol production. This yeast species has shown promise as a bio-active compound for the control of natural infections and postharvest blue mold on mandarin fruit (Wang et al., 2021). Additionally, it has been utilized for malic acid production (Rattanapatpokin et al., 2019), indicating its potential for other biotechnological applications. The use of *M. guilliermondii* in xylitol production offers the advantage of a non-pathogenic microorganism, which reduces the risk of infections associated with *Candida* sp. This is particularly important in industrial settings where strict safety measures and regulations are in place.

Furthermore, the bioactive properties exhibited by *M. guilliermondii* in the control of fruit infections highlight its potential as a beneficial microorganism in agricultural and postharvest applications. Considering both the high xylitol production capability and the non-pathogenic nature of *M. guilliermondii*, it emerges as a promising alternative to *Candida* sp. in the xylitol production process.

Further research and optimization of fermentation conditions using *M. guilliermondii* can be conducted to explore its full potential in industrial-scale xylitol production. This approach would not only ensure high yields of xylitol but also prioritize safety and minimize the risks associated with pathogenic microorganisms.

Table 4.2 Comparison of xylitol producing microorganisms and sequencing information.

Microbial isolated	Yield (g/g)	ITS rDNA and D1/D2 domain of the 26S rDNA	Accession Number
5101A	0.535	*Candida tropicalis*	OR084110
542C	0.591	*Candida tropicalis*	OR084105
562B	0.564	*Candida tropicalis*	OR084104
591A	0.556	*Candida tropicalis*	OR084108
571B	0.541	*Candida tropicalis*	OR084106
561A	0.497	*Candida tropicalis*	OR084107
581A	0.521	*Meyerozyma carpophila*	OR084109
C512G	0.556	*Meyerozyma carpophila*	OR084113
C511C	0.623	*Meyerozyma guilliermondii*	OR084112
C511D	0.587	*Meyerozyma guilliermondii*	OR084111

4.3 Optimization of pretreatment using response surface methodology (RSM) by Box- Behnken

The optimization process followed the design matrix is provided in Table 4.3. The amount of xylose concentration was recorded following the completion of each run (Table 4.4). At 20 minutes, 120 °C, 7.5% H_2SO_4, and 12.5% GCH loading, the highest xylose concentration was observed to be 25.85 g/L (Run; 7).

Table 4.3 The Design Expert's run matrix for maximizing significant variables. X_1-Time; X_2- Temperature; X_3-Acid; X_4- Solid. Concentration of xylose from green coconut husk (g/L).

Runs	(X_1) Time	(X_2) Temperature	(X_3) Acid	(X_4) Solid	Xylose observed value (g/L)	Xylose predicted value (g/L)
1	20	130	7.5	20.0	20.03	20.10
2	30	120	0.0	12.5	11.09	11.02
3	20	110	7.5	20.0	19.79	19.68
4	30	120	7.5	20.0	19.92	20.05
5	30	120	15	12.5	18.99	18.66
6	20	110	0.0	12.5	10.03	10.03
7	20	120	7.5	12.5	25.85	25.82
8	10	120	0.0	12.5	10.89	11.09
9	20	130	7.5	5.0	18.02	18.01
10	20	120	15	20.0	20.02	19.99
11	10	120	7.5	20.0	19.02	19.05
12	30	130	7.5	12.5	19.09	19.10
13	20	120	15.0	5.0	18.59	18.65
14	10	120	15.0	12.5	19.95	19.89
15	20	130	15.0	12.5	19.89	20.04
16	10	120	7.5	5.0	17.22	17.25
17	20	120	7.5	12.5	25.71	25.82
18	20	120	0.0	20.0	13.99	13.89
19	10	110	7.5	12.5	18.00	17.96
20	20	110	7.5	5.0	15.06	14.86
21	20	120	7.5	12.5	25.74	25.82
22	20	110	15	12.5	18.99	19.19
23	30	120	7.5	5.0	14.82	14.94

24	10	130	7.5	12.5	19.09	18.92
25	20	130	0.0	12.5	12.80	12.76
26	30	110	7.5	12.5	16.33	16.47
27	20	120	0.0	5.0	8.33	8.32

The model is accurate in predicting xylose concentration as evidenced by its effective higher F value of 1171.02 and *p*-value of less than 0.05 with lack of fit displaying insignificance. The model predicted value greater than 0.1000 indicates that the model terms are not significant when the difference significantly variable with a *p*-value less than 0.05 was considered.

Table 4.4 Analysis of ANOVA and significance level of the Response Surface Linear model exhibiting GCH hydrolysate for xylose.

Source	Sum of square	Mean square	F-value	p-value	
Model	529.07	37.79	1171.02	< 0.0001	significant
A-Time	1.29	1.29	39.93	< 0.0001	significant
B-Temperature	9.58	9.58	296.78	< 0.0001	significant
C-Acid	202.55	202.55	6276.43	< 0.0001	significant
D-Solid	35.81	35.81	1109.79	< 0.0001	significant
Residual	0.3873	0.0323			
Lack of Fit	0.3789	0.0379	9.02	0.1038	not significant
Pure Error	0.0084	0.0042			
Cor Total	529.46				

Thus, xylose concentration is significantly influenced by the pretreatment conditions of time, temperature, acid concentration, and GCH loading. The xylose concentration has not been significantly impacted by other coupled interaction situations, nevertheless. The following equation was produced when the collected data were submitted to linear regression using Design-Export software (Kavya & Nadumane, 2023). The predicted value of xylose concentration can be calculate following this equation;

Y = -564.13007 + 0.965687 Time + 9.00535 Temperature + 3.37511 Acid + 3.00562 Solid + 0.004178 Time * Temperature - 0.003870 Time * Acid + 0.011012 Time * Solid - 0.006229 Temperature * Acid - 0.009078 Temperature * Solid - 0.018770 Acid * Solid - 0.040210 (Time)² - 0.036831 (Temperature)² - 0.117857 (Acid)² - 0.070616 (Solid)² (4.2)

In RSM 3D surface plots produced by Design expert software, the Regression equation of relevant parameters on sugar concentration has been visually displayed, showing the impact and relationship between various significant variables on xylose concentrations (Figure 4.4 A–F). The obtained graphs were drawn using combination parameters, with the remaining parameters held constant at the midpoint. The optimal assay setting was achieved using the Response Surface Optimizer in Design Expert software, which gave the optimal parameter as time 20 min, temperature 121 °C, H_2SO_4 9%, and GCH loading 12%. This method of determining the optimum value using the response graph can be a little complicated.

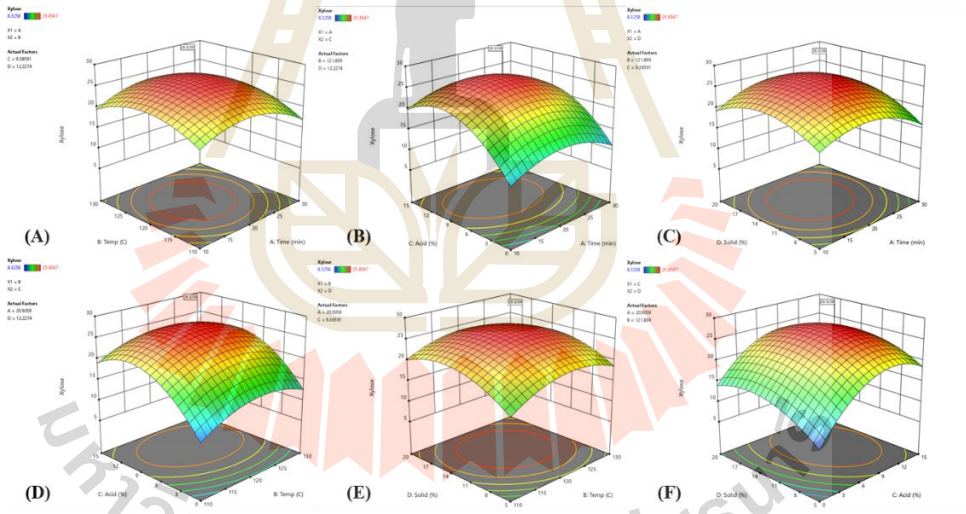

Figure 4.4 RSM 3D surface plots created using Design-Expert software showing the influence and correlation of many important variables on xylose. A) Temperature vs Time, B) Acid vs Time, C) Solid vs Time, D) Acid vs Temperature E) Solid vs Temperature, and F) Solid vs Acid.

Table 4.5 The run matrix obtained by the Design Expert for optimizing significant variables. X_1- Time; X_2- Temperature; X_3-Acid; X_4- Solid. Concentration of xylose from sugarcane bagasse (g/L).

Runs	(X_1) Time	(X_2) Temperature	(X_3) Acid	(X_4) Solid	Xylose observed value (g/L)	Xylose predicted value (g/L)
1	20	120	7.5	12.5	28.32	28.39
2	20	130	7.5	20.0	23.50	23.36
3	20	110	7.5	5.0	17.63	17.65
4	10	120	15.0	12.5	22.52	22.41
5	30	120	0.0	12.5	13.22	13.22
6	20	130	0.0	12.5	15.37	15.30
7	30	130	7.5	12.5	21.66	21.85
8	10	120	7.5	20.0	21.60	21.67
9	20	120	15.0	20.0	22.60	22.82
10	20	130	7.5	5.0	20.60	20.67
11	20	120	7.5	12.5	28.35	28.39
12	30	120	7.5	5.0	17.39	17.34
13	30	110	7.5	12.5	18.91	18.91
14	20	120	7.5	12.5	28.43	28.39
15	10	130	7.5	12.5	21.66	21.75
16	20	120	0.0	20.0	16.56	16.48
17	20	110	7.5	20.0	22.36	22.17
18	30	120	7.5	20.0	22.50	22.60
19	30	120	15.0	12.5	21.56	21.32
20	20	110	0.0	12.5	12.60	12.77
21	10	120	7.5	5.0	19.79	19.72
22	20	120	15.0	5.0	21.16	21.33
23	20	120	0.0	5.0	10.90	10.76
24	10	120	0.0	12.5	13.46	13.59
25	20	130	15.0	12.5	23.46	23.33
26	20	110	15.0	12.5	21.56	21.66
27	10	110	7.5	12.5	20.58	20.48

The result in Table 4.6 indicates that the model is accurate in predicting xylose concentration, with an effective higher F value of 1170.40 and a *p*-value less than 0.05 with lack of fit indicating insignificance. The model predicted value greater than 0.1000 indicates that the model terms are not significant when the difference significantly variable with a *p*-value less than 0.05 was taken into consideration.

Table 4.6 Analysis of ANOVA and significance level of the Response Surface Linear model exhibiting SCB hydrolysate for xylose.

Source	Sum of square	Mean square	F-value	p-value	
Model	545.41	38.96	1170.4	< 0.0001	significant
A-Time	1.59	1.59	47.83	< 0.0001	significant
B-Temperature	13.27	13.27	398.77	< 0.0001	significant
C-Acid	214.55	214.55	6445.62	< 0.0001	significant
D-Solid	38.99	38.99	1171.43	< 0.0001	significant
Residual	0.3994	0.0333			
Lack of Fit	0.391	0.0391	9.31	0.1008	not significant
Pure Error	0.0084	0.0042			
Cor Total	545.81				

Y = -530.78457 + 0.997353 Time + 8.53478 Temperature + 2.95936 Acid + 2.63268 Solid + 0.004178 Time * Temperature - 0.002407 Time * Acid + 0.011012 Time * Solid - 0.002895 Temp * Acid -0.006078 Temperature * Solid - 0.01877 Acid * Solid - 0.041367 (Time)² - 0.035064 (Temperature)² -0.117692 (Acid)² - 0.069698 (Solid)² (4.3)

RSM 3D surface plots produced by Design Expert software, which depict the impact and relationship between various significant variables on xylose concentrations, have been used to visually portray the regression equation of

significant parameters on sugar concentration (Figure 4.5 A–F). The obtained graphs were drawn using combination parameters, with the remaining parameters held constant at the midpoint. The optimal assay condition was determined using the Response Surface Optimizer in Design Expert software, which gave the optimal parameter as time 20 min, temperature 121 °C, H_2SO_4 7%, and sugarcane bagasse 15%. This method of determining the optimum value using the response graph is a little bit tricky.

The previous work reported on a variety of pretreatment methods for lignocellulose material, including microbial pretreatment, enzymatic pretreatment, organic acid pretreatment, and inorganic salt pretreatment (Zhou & Tian, 2022). The pretreatment method using acid and steam explosion has an advantage over other approaches, though, as it is known to maximize sugar recovery from lignocellulosic biomass. They successfully disassemble biomass' intricate structure, enabling the release of sugars that can then be processed into useful goods like xylitol. For

industrial applications, pretreatment methods using acid and steam diffusion may be both economically feasible and easily scalable. They have been found to be compatible with a variety of lignocellulosic feedstocks, including agricultural residues, wood chips, and energy crops, making them appealing options for large-scale xylitol production. They also require minimal equipment and can be integrated into existing biomass processing facilities. Due to its adaptability, xylitol may be produced from a variety of biomass sources (Isikgor & Becer, 2015). In a study by Morais et al. (2023), it was observed that employing a lower acid concentration (0.5% H_2SO_4) with a longer reaction time (100 min) resulted in a significantly higher xylose concentration in the hydrolysate (90.32 g/L). Conversely, utilizing a higher acid concentration (7% H_2SO_4) with a shorter reaction time (20 min) led to a comparatively lower xylose concentration of 26.32 g/L. These findings suggest that a longer reaction time combined with a lower acid concentration favors the hydrolysis of hemicellulose sugars, leading to an increased release of xylose. This higher xylose concentration is particularly advantageous in the context of xylitol production, as xylose serves as a crucial precursor. However, it should be noted that the approach involving a shorter reaction time and higher acid concentration may have other benefits, such as reduced formation of inhibitory compounds or improved process efficiency. These results

underscore the importance of carefully selecting the appropriate pretreatment conditions for biomass hydrolysis, considering factors such as acid concentration, reaction time, and the desired product yield. Optimal conditions may vary depending on specific objectives, including xylose yield, inhibitory compound formation, process efficiency, and subsequent fermentation considerations (Isikgor & Becer, 2015).

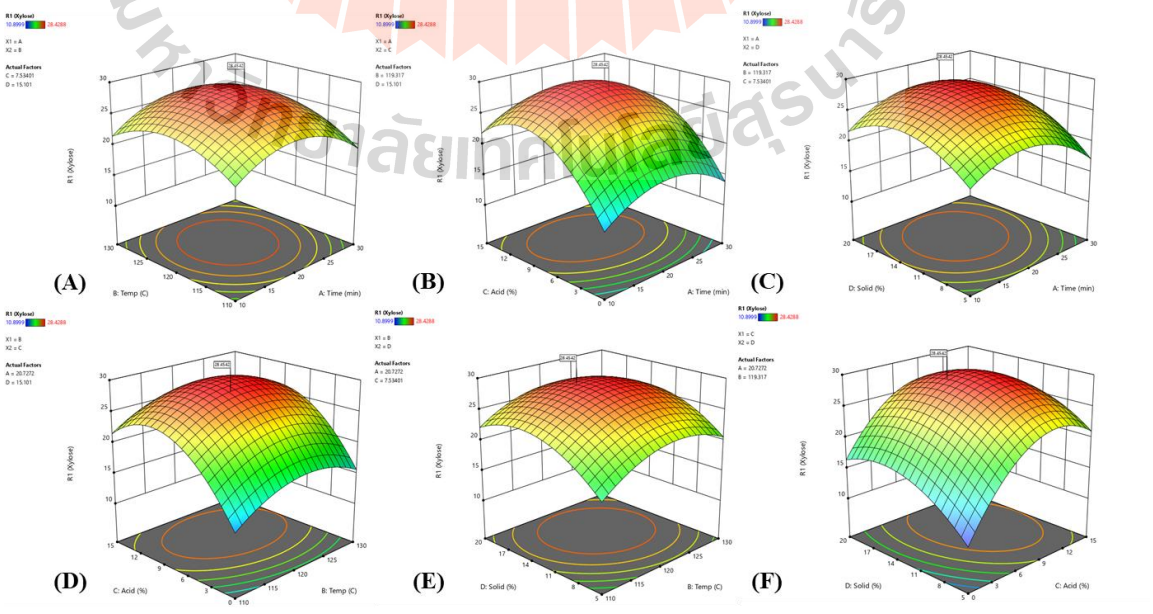

Figure 4.5 RSM 3D surface plots created using Design-Expert software showing the influence and correlation of many important variables on xylose. A) Temperature vs Time, B) Acid vs Time, C) Solid vs Time, D) Acid vs Temperature, E) Solid vs Temperature, and F) Solid vs Acid.

The comparison between SCB hydrolysate and GCH hydrolysate was analyzed using an independent samples t-test. The mean values and standard deviations for each group were calculated. The mean value of SCB hydrolysate was 24.10, with a standard deviation of 2.87. On the other hand, GCH hydrolysate had a mean value of 21.42, and a standard deviation of 2.90. The calculated t-value (*t-cal*) was 1.97, while the critical t-value (*t-crit*) was at a significance level of 0.05 and with 18 degrees of freedom (*df*) was 2.10. Based on the statistical analysis, the *p*-value obtained was 0.065. Although the *p*-value is greater than the commonly used significance level of 0.05, indicating that the difference between the means is not statistically significant, it is worth noting that there might still be a trend or a potential relationship between the two groups. Further investigation with a larger sample size could provide more insights into the differences between SCB hydrolysate and GCH hydrolysate. The result is shown in Table 4.7.

Table 4.7 Comparison of Mean Values and t-test Results for SCB Hydrolysate and GCH Hydrolysate.

Source	n	Mean	SD	t-cal	t-crit	df	p
SCB hydrolysate	10	24.10	2.87	1.97	2.10	18	.065
GCH hydrolysate	10	21.42	2.90				

Pretreatment of lignocellulosic biomass offers a significant deal of potential to increase efficiency and reduce production costs through research and development (Li et al., 2020). The overall cost of using lignocellulose for practical applications will be significantly reduced by combining several biomass pretreatment techniques with other procedures such enzymatic saccharification, detoxification, fermentation of the hydrolysates, and product recovery. Thus, advancements in pretreatment technologies, microorganisms that produce cellulolytic enzymes, the best possible utilization of the components of biomass, and process integration are anticipated to be key factors in the economic success of lignocellulosic conversion in the future (Singh et al., 2021).

4.4 Cultivation test

For a comparative analysis, *M. guilliermondii C511C* was further cultivated in two different mediums: medium A (non-detoxified GCH hydrolysate) and medium B (detoxified GCH hydrolysate). The results showed that both the cell growth rate and xylitol production were lower in medium A with batch culture than those in medium containing detoxified hydrolysate (medium B). The result of cultivation of xylitol shown in Table 4.9 and the highest values of the specific growth rate (OD_{600} = 3.96 ± 0.04), and xylitol (14.61 g/L) on substrate (Figure 4.6).

Table 4.8 Comparison of xylitol produced by *M. guilliermondii* C511C with non-detoxified GCH hydrolysate and detoxified GCH hydrolysate after 42 h of cultivation.

Modified medium	OD_{600}	Xylose (g/L)	Xylitol (g/L)	Yield (g/g)
Non-detoxified GCH hydrolysate	3.27 ± 0.07	1.66 ± 0.03	12.50 ± 0.01	0.56
Detoxified GCH hydrolysate	3.95 ± 0.04	0.95 ± 0.03	14.61 ± 0.10	0.65

Detoxification with activated carbon following acid pretreatment offers several benefits in biomass processing. Firstly, activated carbon has a high adsorption capacity, which enables it to effectively remove inhibitory compounds such as furfural, hydroxy-methyl-furfural (HMF), organic acids, and phenolic compounds. These inhibitory compounds can have detrimental effects on subsequent fermentation processes by inhibiting the growth and metabolism of microorganisms, leading to decreased product yields. Detoxification helps to mitigate these inhibitory effects and improve the overall efficiency of downstream fermentation. Furthermore, activated carbon can also aid in the removal of residual acids or acid salts remaining in the hydrolysate after acid pretreatment. These acidic components may have adverse effects on the fermentation process and the viability of microorganisms. Activated carbon acts as an adsorbent and effectively reduces the acidity of the hydrolysate, creating a more favorable environment for fermentation. In addition to its detoxification capabilities, activated carbon can contribute to the purification of the hydrolysate by removing impurities, colorants, and unwanted odors, thereby enhancing the quality of the final product. This purification step is particularly important when the hydrolysate is intended for further downstream applications or as a feedstock for value-added products. Overall, the detoxification of acid-pretreated biomass using activated carbon provides benefits such as the removal of inhibitory

compounds, reduction of acidity, and purification of the hydrolysate. These advantages contribute to improving the feasibility and efficiency of subsequent fermentation processes and the quality of the final product (Abdul Manaf et al., 2022; Prakash et al., 2011).

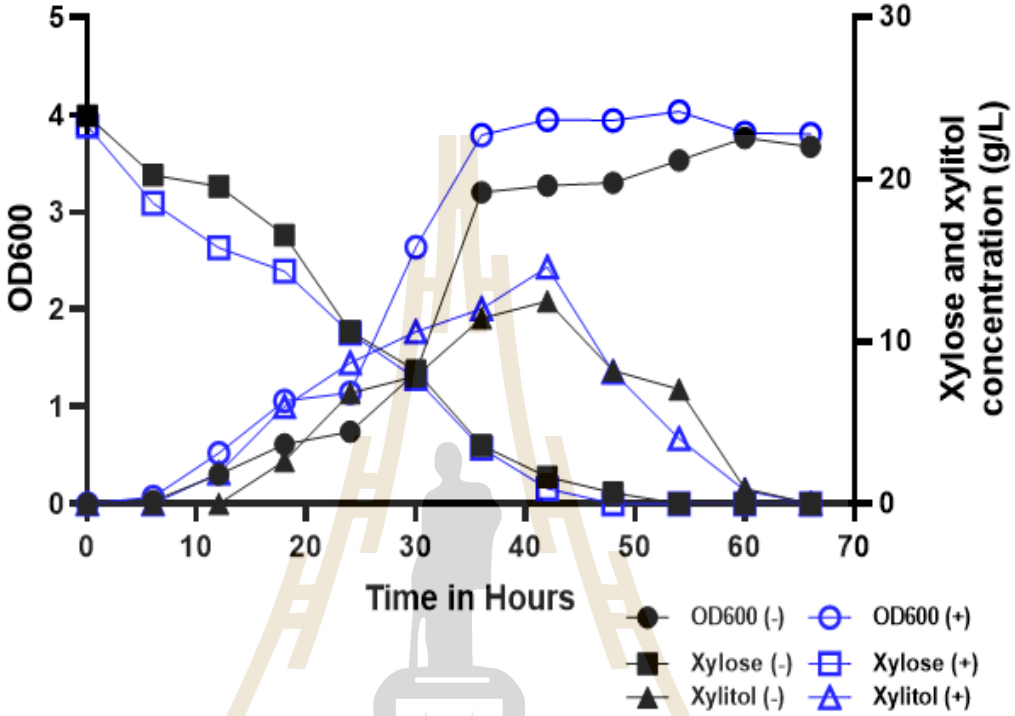

Figure 4.6 Cultivation of xylitol with (-) non-detoxified GCH hydrolysate and (+) detoxification GCH hydrolysate with 3% (w/v) activated carbon for 1 h.

4.5 Optimization of media using respond surface methodology (RSM) by Box- Behnken

Fermentation was performed in accordance with the design matrix shown in Table 4.10. The quantity of xylitol produced with each run was noted in the supporting materials afterward (Table 4.11).

Table 4.9 The run matrix obtained by the Design Expert for optimizing significant variables. X_1- Xylose; X_2- Yeast extract; X_3- KH_2PO_4; X_4- $(NH_4)_2SO_4$. And concentration of xylitol (g/L).

Runs	(X_1) Xylose	(X_2) Yeast extract	(X_3) KH_2PO_4	(X_4) $(NH_4)_2SO_4$	Xylitol observed value (g/L)	Xylitol predicted value (g/L)
1	20	1	0	1	9.1	9.36
2	20	3	1.5	1	13.66	13.76
3	30	3	1.5	2	20.87	20.63
4	20	3	1.5	1	13.75	13.76
5	10	3	3	1	4.81	4.94
6	20	3	0	0	6.21	6.13
7	20	3	1.5	1	13.87	13.76
8	30	3	1.5	0	10.03	10.17
9	20	3	0	2	11.02	11.27
10	20	3	3	0	10.85	10.46
11	20	5	1.5	0	9.85	10.07
12	30	3	0	1	12.54	12.47
13	20	3	3	2	14.49	14.42
14	10	3	1.5	0	4.48	4.8
15	20	1	1.5	2	14.02	13.87
16	20	5	0	1	9.12	8.9
17	10	5	1.5	1	4.5	4.39
18	10	3	1.5	2	3.52	3.45
19	20	1	1.5	0	8.85	8.63
20	20	5	3	1	14.03	13.85
21	10	3	0	1	2.2	2.06
22	10	1	1.5	1	4.87	4.73
23	30	1	1.5	1	14.95	14.91
24	20	1	3	1	11.6	11.9
25	30	3	3	1	16.87	17.08
26	20	5	1.5	2	13.65	13.94
27	30	5	1.5	1	16.76	16.76

Table 4.10 Analysis of ANOVA and significance level of the Response Surface Linear model exhibiting xylitol production.

Source	Sum of square	Mean square	F-value	p-value

Model	572.18	40.87	500.39	< 0.0001	significant
A-Xylose	381.07	381.07	4665.64	< 0.0001	significant
B-Yeast extract	1.69	1.69	20.73	0.0007	significant
C-KH_2PO_4	42.05	42.05	514.87	< 0.0001	significant
D-$(NH_4)_2SO_4$	62.16	62.16	761.06	< 0.0001	significant
Residual	0.9801	0.0817			
Lack of Fit	0.9567	0.0957	8.16	0.1141	not significant
Pure Error	0.0235	0.0117			
Cor Total	573.16				

The statistical analysis using Analysis of Variance (ANOVA) revealed significant effects of the factors on the response variable. The overall model was found to be highly significant (F = 500.39, p < 0.0001), indicating that the factors collectively explain a substantial amount of the variation in the response variable. Individually, all four factors were found to have statistically significant effects on the response variable. The Xylose factor (F = 4665.64, p < 0.0001), Yeast extract factor (F = 20.73, p = 0.0007), KH_2PO_4 factor (F = 514.87, p < 0.0001), and $(NH_4)_2SO_4$ factor (F = 761.06, p < 0.0001) were each associated with significant variation in the response variable. These results indicate that the manipulation of these factors has a significant impact on the outcome. The regression equation, Y = - 9.68255 + 1.22879 Xylose + 0.786492 Yeast extract + 2.8136 KH_2PO_4 - 0.249837 $(NH_4)_2SO_4$ + 0.027312 Xylose * Yeast extract + 0.028767 Xylose * KH_2PO_4 + 0.2951 Xylose * $(NH_4)_2SO_4$ + 0.200417 Yeast extract * KH_2PO_4 - 0.171162 Yeast extract * $(NH_4)_2SO_4$ - 0.1965 KH_2PO_4 * (NH4)2SO4 - 0.027136 $(Xylose)^2$ - 0.2124 $(Yeast\ extract)^2$ - 0.848561 $(KH_2PO_4)^2$ -1.28398 $(NH_4)_2SO_4$, provides a quantitative relationship between the variables and xylitol concentration.

To visually depict the impact and relationship between the significant variables and xylitol synthesis, Response Surface Methodology (RSM) 3D surface plots were generated using Design Expert software. These plots, shown in Figure 4.7 A–F, illustrate the effects of varying the concentrations of xylose, yeast extract, KH_2PO_4, and $(NH_4)_2SO_4$ on xylitol production. The remaining parameters were held constant at their midpoint values during the plot generation. To determine the optimal assay conditions for xylitol production, the Response Surface Optimizer in Design Expert software was employed. The optimized parameters were found to be xylose at 30 g/L, yeast extract at 3 g/L, KH_2PO_4 at 2 g/L, and $(NH_4)_2SO_4$ at 2 g/L. These values represent the combination of variables that maximizes the predicted xylitol concentration. It is important to note that determining the optimal values using response graphs and optimization algorithms can be somewhat sophisticated.

However, these methods provide valuable insights into the relationship between variables and offer guidance for process optimization and enhanced xylitol yield. The findings of this study contribute to a deeper understanding of the fermentation conditions influencing xylitol production and can serve as a basis for further research and development in this field (Vardhan et al., 2022).

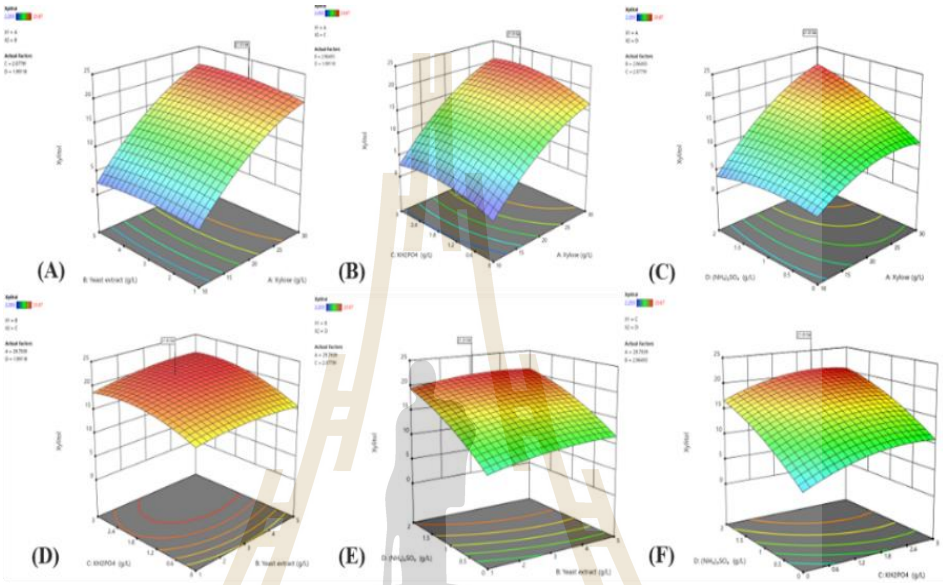

Figure 4.7 Utilizing Design-Expert software, RSM 3D surface plots were created to show the interactions and impacts of many critical variables on xylitol. A) Yeast extract vs Xylose and B) KH_2PO_4 vs Xylose C) $(NH_4)_2SO_4$ vs Xylose and D) KH_2PO_4 vs Yeast extract E) $(NH_4)_2SO_4$ vs Yeast extract and F) $(NH_4)_2SO_4$ vs KH_2PO_4.

4.6 Fed-batch fermentation in 5-L bioreactor

4.6.1 Batch and fed-batch fermentation in 5-L bioreactor with pure xylose.

For comparative analysis, *M. guilliermondii C511C* was subjected to two different fermentation processes: batch fermentation and fed-batch fermentation. The results revealed contrasting outcomes between the two processes. In batch fermentation, the cell growth rate was higher, whereas the xylitol concentration was higher in fed-batch fermentation. Figure 4.8 depicts these results. In the case of batch fermentation, the cell dry weight and accumulated xylitol at 36 h were 2.74 ± 0.08 and 17.16 ± 0.21 g/L, respectively, with a yield of 0.76 g/g. Conversely, in fed-batch fermentation at 84 hours, the cell dry weight and accumulated xylitol were 9.96 ± 0.10 and 41.68 ± 0.38 g/L, respectively, with a yield of 0.68 g/g.

Fed-batch fermentation is commonly employed to enhance the productivity of microbial cultures over conventional batch fermentation. However, this process is more complex and requires advanced control strategies to optimize

culture conditions. Several variables can impact the performance of fed-batch fermentation, potentially leading to reduced yields. The feeding rate, for instance, should be carefully optimized to ensure sufficient nutrient availability for microbial growth and productivity. If the feed rate is too low or too high, it may result in lower yield and growth inhibition. Additionally, oxygen limitation can affect xylitol production and lead to decreased yield, as oxygen is crucial for microbial growth and metabolism (Tamburini et al., 2015).

4.6.2 Fed-batch fermentation of detoxified GCH hydrolysate

The result is shown in Figures 4.8C. *M. guilliermondii C511C* was utilized for xylitol production during the 5-L bioreactor. The optimum process conditions for xylitol production were 30 °C, pH 5.5, 300 rpm of agitation, and aeration rate at 1 vvm. The composition of medium used for fermentation is 30 g/L of xylose from the detoxified GCH hydrolysate, 3 g/L yeast extract, 2 g/L KH_2PO_4, 5 g/L glucose, 0.5 g/L $MgSO_4$ and 2 g/L $(NH_4)_2SO_4$. During the fermentation, xylose from the detoxified GCH hydrolysate was added. The highest xylitol yield obtained at 96 h was 0.70 g/g (28.19 ± 0.46 g/L).

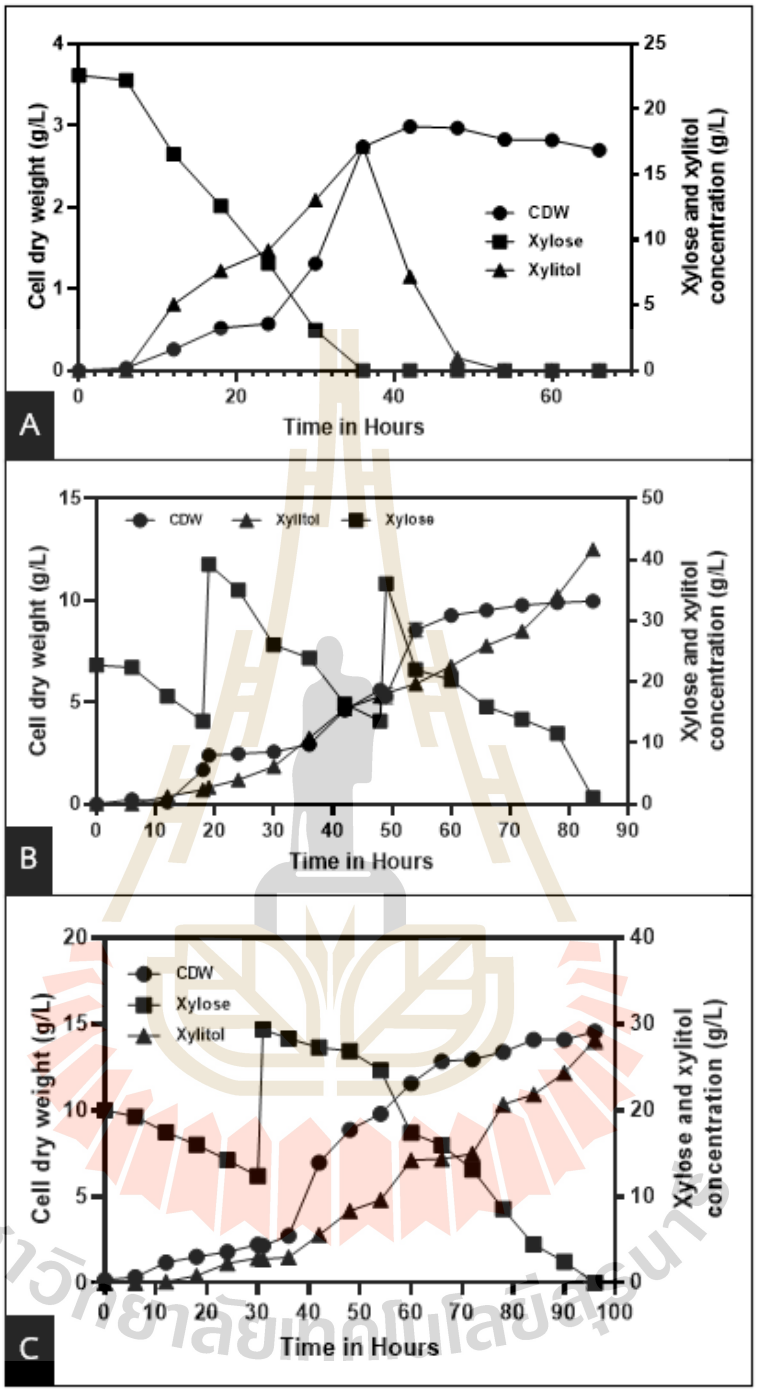

Figure 4.8 Fermentation of *M. guilliermondii* C511C in 5-L bioreactor (A) batch fermentation with pure xylose (B) fed-batch fermentation with pure xylose and (C) fed-batch fermentation with GCH hydrolysate after detoxification.

Table 4.11 Comparison of microorganisms for xylitol production with different sources and processes.

Microorganism	Feedstock	Processing method	Scale (L)	Xylitol titer (g/L)	Xylitol yield (g/g)	Ref
M. guilliermondii C511C	green coconut husk	9% H_2SO_4 at 121 °C for 20 min + 3% (w/v) activated carbon (AC) for 1 h at 30 °C for 200 rpm	3.5	28.19 ± 0.46	0.70	This study
P. caribbica MTCC 5703	Corncob	3.76% H_2SO_4 at 140 °C for 90 min + 3% (w/v) AC, pH 4.5 for 15 min	5.0	124.1 ± 0.45	0.80 ± 0.02	(Dasgupta et al., 2022)
P. fermentans	Sugarcane bagasse	2% (w/v) H_2SO_4 with 50% (w/v) solid loading at 125 °C for 30 min	2.5	86.6	0.75	(Narisetty et al., 2021)
Kluyveromyces marxianus ATCC 36907	Oil palm fronds	5.3% CH_3COOH at 100 °C for 74 min + 3% AC	-	10.15	0.34	(Abdul Manaf et al., 2022)
M. guilliermondii F22 and S. cerevisiae	Rice straw	2.5% H_2SO_4 at 100 °C for 30 min + over liming with CaO 30 min follow by 3% AC	14.0	25.8	0.60	(Singh et al., 2021)
C. tropicalis GS18	Rice straw	1% HNO_3 at 121 °C for 30 min + 2% (w/v) AC	-	34.21	0.60	(Kaur et al., 2022)
M. caribbica InaCC Y67	Sugarcane trash	2% $C_2H_2O_4$, Solid and liquid ratio (1:10) at 121 °C for 60 min follow by 180 °C for 7.5 min with microwave.	-	6.49 ± 0.12	0.27 ± 0.01	(Pramasari et al., 2023)
Pachysolen tannophilus MTTC 1077	Corncob	10 % Solid loading with 0.1% (w/v) H_2SO_4 at 120 °C for 1 h	-		0.80	(Ramesh et al., 2013)

From Table 4.12, The outcome of this study is in range with the work of (Kaur et al., 2022) using *C. tropicalis* GS18. Moreover, production of xylitol using other microorganisms in the previous studies have been reported in the range of 0.27-0.80 g/g of xylitol yield (Durairaj et al., 2004; Pramasari et al., 2023). The fermentation ability of *M. guilliermondii C511C* cells can further be optimized by adaptation. The use of pure xylose for inoculum growth as well as xylitol production in the control media as a limiting factor due to high cost and availability as compared to other

carbon source. Therefore, the utilization of xylose provided by detoxified GCH hydrolysate not only yields a respectable amount of xylitol but also offers a more cost-effective option.

In this study, a total of 28.19 ± 0.46 g/L xylitol with 0.70 g/g yield was produced using 40 g/L xylose from detoxified GCH hydrolysate after 96 h of fermentation as shown in figure 4.9.

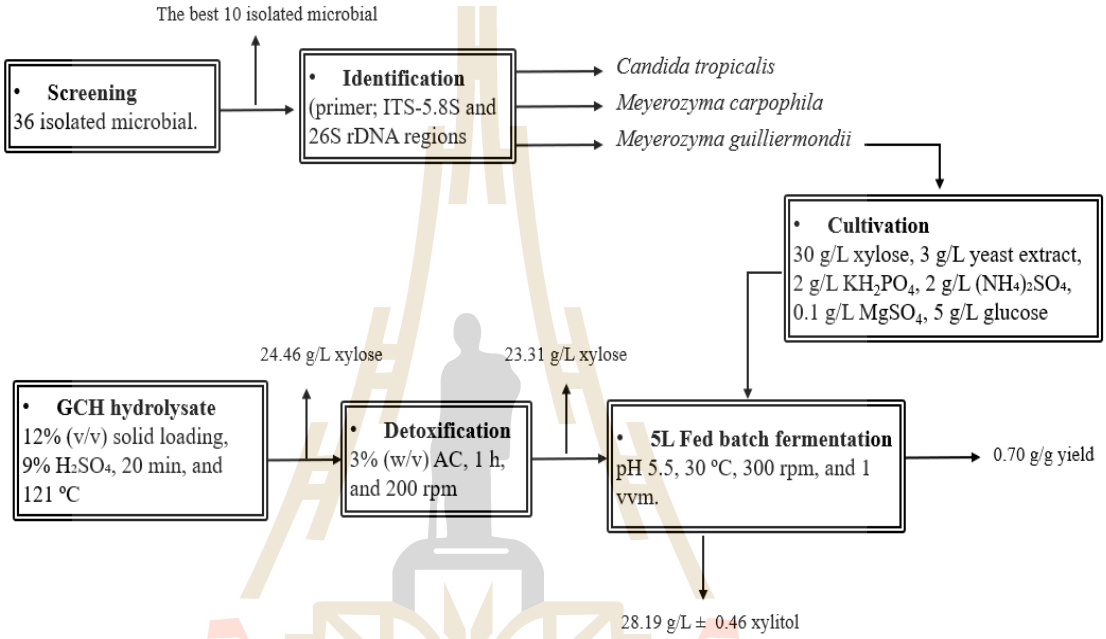

Figure 4.9 Process of xylitol fermentation by *M. guilliermondii* C511C with GCH hydrolysate.

CHAPTER 5
CONCLUSION

Characterization of studies that the isolated yeast stains 5101A, 542C, 562B, 591A, 571B and 561A belong to *Candida tropicalis*. The isolated yeast strains 581A and C512G belong to *Meyerozyma carpophila* and the isolated yeast strains C511C and C511D belong to *Meyerozyma guilliermondii*. The optimization of the pretreatment of GCH hydrolysate parameters (time, temperature, acid concentration, and solid loading) gave a considerable increase in xylose concentration from 8.33-25.85 g/L. According to ANOVA analysis, the optimization of the fermentation parameters (xylose, yeast extract, KH_2PO_4, $(NH_4)_2SO_4$) provided a considerable increase in xylitol production from 2.20-20.87 g/L. For 5-L bioreactor with fed-batch fermentation, *M. guilliermondii* C 5 1 1 C can produce xylitol by detoxified GCH hydrolysate with 0.70 g/g yield (28.19 ± 0.46 g/L). Additionally, this isolated C511C yeast showed industrial potential for producing xylitol in a variety of environmental conditions. Finally, the current study demonstrated that *M. guilliermondii* C511C may be scaled up for industrial synthesis of xylitol using detoxified GCH hydrolysate, the process in this research proved to be simple and promising.

REFERENCES

REFERENCES

Abdul Manaf, S. F., Indera Luthfi, A. A., Md Jahim, J., Harun, S., Tan, J. P., & Mohd Shah, S. S. (2022). Sequential detoxification of oil palm fronds hydrolysate with coconut shell activated charcoal and pH controlled in bioreactor for xylitol production. *Chemical Engineering Research and Design, 179*, 90-106.

Ahuja, V., Macho, M., Ewe, D., Singh, M., Saha, S., & Saurav, K. (2020). Biological and Pharmacological Potential of Xylitol: A Molecular Insight of Unique Metabolism. *Foods, 9*(11).

Ajala, E. O., Ighalo, J. O., Ajala, M. A., Adeniyi, A. G., & Ayanshola, A. M. (2021). Sugarcane bagasse: a biomass sufficiently applied for improving global energy, environment and economic sustainability. *Bioresources and Bioprocessing, 8*(1), Article 87.

Bevilaqua, G. C., Maugeri Filho, F., & Forte, M. B. S. (2023). Simultaneous production of xylitol and arabitol by *Candida tropicalis* fermentation improving agro-industrial wastes valorization. *Food and Bioproducts Processing, 140*, 29-45.

Box, G. E. P., & Draper, N. R. (1959). A Basis for the Selection of a Response Surface Design. *Journal of the American Statistical Association, 54*(287), 622-654.

Chen, S. J., Chen, X., & Zhu, M. J. (2022). Xylose recovery and bioethanol production from sugarcane bagasse pretreated by mild two-stage ultrasonic assisted dilute acid. *Bioresour Technol, 345*, 126463.

Cingi, C., Birdane, L., Ural, A., Oghan, F., & Bal, C. (2014). Comparison of nasal hyperosmolar xylitol and xylometazoline solutions on quality of life in patients with inferior turbinate hypertrophy secondary to nonallergic rhinitis. *Int Forum Allergy Rhinol, 4*(6), 475-479.

Dasgupta, D., Bandhu, S., Adhikari, D. K., & Ghosh, D. (2017). Challenges and prospects of xylitol production with whole cell bio-catalysis: A review. *Microbiol Res, 197*, 9-21.

Dasgupta, D., Sidana, A., Sarkar, B., More, S., Ghosh, D., Bhaskar, T., & Ray, A. (2022). Process development for crystalline xylitol production from corncob biomass by *Pichia caribbica*. *Food and Bioproducts Processing, 133*, 45-56.

de Freitas Branco, R., dos Santos, J. C., & da Silva, S. S. (2011). A novel use for sugarcane bagasse hemicellulosic fraction: Xylitol enzymatic production. *Biomass and Bioenergy, 35*(7), 3241-3246.

Durairaj, L., Launspach, J., Watt, J. L., Businga, T. R., Kline, J. N., Thorne, P. S., & Zabner, J. (2004). Safety assessment of inhaled xylitol in mice and healthy volunteers. *Respir Res, 5*(1), 13.

Ezemba, C., Ekwagbalu, E., A.,, & Ezemba, A., S.,. (2022). Fermentation, type of fermentation, design & uses of fermenter and optimizaion of fermentation process. *Researchgate*.

Gasmi Benahmed, A., Gasmi, A., Arshad, M., Shanaida, M., Lysiuk, R., Peana, M., Pshyk-Titko, I., Adamiv, S., Shanaida, Y., & Bjorklund, G. (2020). Health benefits of xylitol. *Appl Microbiol Biotechnol, 104*(17), 7225-7237.

Gong, Y., Zhang, Q., Qiao, L., Lv, D., Ruan, J., Chen, H., Gong, J., & Shi, G. (2015). Xylitol Gum Chewing to Achieve Early Postoperative Restoration of Bowel Motility After Laparoscopic Surgery. *Surg Laparosc Endosc Percutan Tech, 25*(4), 303-306.

Gosalawit, C., Imsoonthornruksa, S., Gilroyed, B. H., McNea, L., Boontawan, A., & Ketudat-Cairns, M. (2021). The potential of the oleaginous yeast *Rhodotorula paludigena* CM33 to produce biolipids. *J Biotechnol, 329*, 56-64.

Gosalawit, C., Imsoonthornruksa , S., Udomsil , N., & Ketudut , M., C. (2020). Genome Sequence of the Oleaginous Yeast *Rhodotorula paludigena* Strain CM33, a Potential Strain for Biofuel Production. *Microbiology Resource Announcements, 9*(19).

Hernandez-Mejia, C., Gnanakumar, E. S., Olivos-Suarez, A., Gascon, J., Greer, H. F., Zhou, W., Rothenberg, G., & Raveendran Shiju, N. (2016). Ru/TiO_2-catalysed hydrogenation of xylose: The role of the crystal structure of the support. *Catalysis Science and Technology, 6*(2), 577-582.

Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry, 6*(25), 4497-4559.

Kaur, S., Guleria, P., Sidana, A., & Yadav, S. K. (2022). Efficient process for xylitol production from nitric acid pretreated rice straw derived pentosans by *Candida tropicalis* GS18. *Biomass and Bioenergy, 166*.

Kavya, D., & Nadumane, V. K. (2023). Enhanced l-Methioninase production by *Methylobacterium* sp. JUBTK33 through RSM and its anticancer potential. *Biocatalysis and Agricultural Biotechnology, 47*.

Kiet A. Ly, M., MPH, Peter Milgrom, D., & Marilynn Rothen, B., RDH. (2006). Xylitol, Sweeteners, and Dental Caries. *Pediatric Dentistry, 28*(2), 154-163.

Kim, J. H., Han, K. C., Koh, Y. H., Ryu, Y. W., & Seo, J. H. (2002). Optimization of fed-batch fermentation for xylitol production by *Candida tropicalis*. *J Ind Microbiol Biotechnol, 29*(1), 16-19.

Kumar, S., Dheeran, P., Singh, S. P., Mishra, I. M., & Adhikari, D. K. (2015). Bioprocessing of bagasse hydrolysate for ethanol and xylitol production using thermotolerant yeast. *Bioprocess Biosyst Eng, 38*(1), 39-47.

Li, Y., Qi, B., & Wan, Y. (2020). Separation of monosaccharides from pretreatment inhibitors by nanofiltration in lignocellulosic hydrolysate: Fouling mitigation by activated carbon adsorption. *Biomass and Bioenergy, 136*.

Looke, M., Kristjuhan, K., & Kristjuhan, A. (2011). Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques, 50*(5), 325-328.

Lugani, Y., Singla, R., & Singh Sooch, B. (2015). Optimization of Cellulase Production from Newly Isolated *Bacillus* Sp. Y3. *Journal of Bioprocessing & Biotechniques, 5*(11).

Lugani, Y., & Sooch, B. S. (2020). Fermentative production of xylitol from a newly isolated xylose reductase producing *Pseudomonas putida* BSX-46. *Lwt, 134*.

Minihane, B., J., & Brown, D., E.,. (1986). Fed-batch fermentation. *Biotech, 4*, 207-218.

Mohamad, N. L., Mustapa Kamal, S. M., & Mokhtar, M. N. (2015). Xylitol Biological Production: A Review of Recent Studies. *Food Reviews International, 31*(1), 74-89.

Mun, L. W., Rafiqul, I. S. M., Sakinah, A. M. M., & Zularisam, A. W. (2016). Purification of bioxylitol by liquid–liquid extraction from enzymatic reaction mixture. *Separation Science and Technology, 51*(14), 2369-2377.

Narisetty, V., Castro, E., Durgapal, S., Coulon, F., Jacob, S., Kumar, D., Kumar Awasthi, M., Kishore Pant, K., Parameswaran, B., & Kumar, V. (2021). High level xylitol production by *Pichia fermentans* using non-detoxified xylose-rich sugarcane bagasse and olive pits hydrolysates. *Bioresour Technol, 342*, 126005.

Pant, S., Ritika, Prakash, A., & Kuila, A. (2022). Integrated production of ethanol and xylitol from Brassica juncea using *Candida sojae* JCM 1644. *Bioresour Technol, 351*, 126903.

Ping, Y., Ling, H.-Z., Song, G., & Ge, J.-P. (2013). Xylitol production from non-detoxified corncob hemicellulose acid hydrolysate by *Candida tropicalis*. *Biochemical Engineering Journal, 75*, 86-91.

Prakash, G., Varma, A. J., Prabhune, A., Shouche, Y., & Rao, M. (2011). Microbial production of xylitol from D-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast *Debaryomyces hansenii*. *Bioresour Technol, 102*(3), 3304-3308.

Pramasari, D. A., Oktaviani, M., Thontowi, A., Purnawan, A., Ermawar, R. A., Sondari, D., Ningrum, R. S., Laksana, R. P. B., Lianawati, A., Fahrezi, M. Z. M., Salsabila, Q., & Hermiati, E. (2023). The use of hemicellulose acid hydrolysate for hydrolysis of sugarcane trash and its fermentation for producing xylitol. *Industrial Crops and Products, 193*.

Ramesh, S., Muthuvelayudham, R., Rajesh Kannan, R., & Viruthagiri, T. (2013). Enhanced Production of Xylitol from Corncob by *Pachysolen tannophilus* Using Response Surface Methodology. *Int J Food Sci, 2013*, 514676.

Rattanapatpokin, T., Tachaapikoon, C., Waeonukul, R., Wattanachaisaereekul, S., Ratanakhanokchai, K., & Pason, P. (2019). Newly isolated malic acid fermenting yeast *Meyerozyma caribbica* AY 33-1 for bioconversion of glucose and cassava pulp. *Science and Engineering, 1*(2435-2993).

Ravindran, R., & Jaiswal, A. K. (2016). A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. *Bioresour Technol, 199*, 92-102.

Salli, K., Lehtinen, M. J., Tiihonen, K., & Ouwehand, A. C. (2019). Xylitol's health benefits beyond dental health: A comprehensive review. *Nutrients, 11*(8), Article 1813.

Sarabia, L. A., & Ortiz, M. C. (2009). Response Surface Methodology. In S. D. Brown, R. Tauler, & B. Walczak (Eds.), *Comprehensive Chemometrics* (pp. 345-390). Elsevier.

Singh, S., Kaur, D., Yadav, S. K., & Krishania, M. (2021). Process scale-up of an efficient acid-catalyzed steam pretreatment of rice straw for xylitol production by *C. Tropicalis* MTCC 6192. *Bioresour Technol, 320*(Pt B), 124422.

Sitepu, I. R., Garay, L. A., Sestric, R., Levin, D., Block, D. E., German, J. B., & Boundy-Mills, K. L. (2014). Oleaginous yeasts for biodiesel: current and future trends in biology and production. *Biotechnol Adv, 32*(7), 1336-1360.

Sukpipat, W., Komeda, H., Prasertsan, P., & Asano, Y. (2017). Purification and characterization of xylitol dehydrogenase with l-arabitol dehydrogenase activity from the newly isolated pentose-fermenting yeast *Meyerozyma caribbica* 5XY2. *J Biosci Bioeng, 123*(1), 20-27.

Tamburini, E., Costa, S., Marchetti, M. G., & Pedrini, P. (2015). Optimized Production of Xylitol from Xylose Using a Hyper-Acidophilic *Candida tropicalis*. *Biomolecules, 5*(3), 1979-1989.

Tian, X., Chen, X., Ma, C., Su, K., Geng, Q., Zhao, F., & Liu, X. (2022). Green synthesis of blue-green photoluminescent β-SiC nanowires with core-shell structure using coconut shell as carbon source. *Ceramics International, 48*(24), 36273-36278.

Vardhan, H., Sasamal, S., & Mohanty, K. (2022). Fermentation process optimisation based on ANN and RSM for xylitol production from areca nut husk followed by xylitol crystal characterisation. *Process Biochemistry, 122*, 146-159.

Vaz de Arruda, P., dos Santos, J. C., de Cássia Lacerda Brambilla Rodrigues, R., da Silva, D. D. V., Yamakawa, C. K., de Moraes Rocha, G. J., Júnior, J. N., da Cruz Pradella, J. G., Vaz Rossell, C. E., & das Graças de Almeida Felipe, M. (2017). Scale up of xylitol production from sugarcane bagasse hemicellulosic hydrolysate by *Candida guilliermondii* FTI 20037. *Journal of Industrial and Engineering Chemistry, 47*, 297-302.

Wang, Z., Li, J., Liu, J., Tian, X., Zhang, D., & Wang, Q. (2021). Management of blue mold (*Penicillium italicum*) on mandarin fruit with a combination of the yeast, *Meyerozyma guilliermondii* and an alginate oligosaccharide. *Biological Control, 152*.

Wannawilai, S., Lee, W. C., Chisti, Y., & Sirisansaneeyakul, S. (2017). Furfural and glucose can enhance conversion of xylose to xylitol by *Candida magnoliae* TISTR 5663. *J Biotechnol, 241*, 147-157.

Yu, H., Lin, Z.-X., Xiang, W.-L., Huang, M., Tang, J., Lu, Y., Zhao, Q.-H., Zhang, Q., Rao, Y., & Liu, L. (2022). Antifungal activity and mechanism of d-limonene against foodborne opportunistic pathogen *Candida tropicalis*. *Lwt, 159*.

Zha, J., Yuwen, M., Qian, W., & Wu, X. (2021). Yeast-Based Biosynthesis of Natural Products From Xylose. *Front Bioeng Biotechnol, 9*, 634919.

Zhou, M., & Tian, X. (2022). Development of different pretreatments and related technologies for efficient biomass conversion of lignocellulose. *Int J Biol Macromol, 202*, 256-268.

VITAE

Miss Paramee Wadduangdee was born on 9th March 1998 in Pathum Thani, Thailand. In 2016, she finished her high school from Kaeng Khoi School, Saraburi, Thailand. In July 2020, she graduated with a bachelor's degree in biotechnology from King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. In October 2020, she started to work as a research assistant at Suranaree University of Technology. In December 2021, she obtianed a scholarship for a double degree Master program from Assoc. Prof. Dr. Apichat Boontawan, Suranaree University of Technology, Thailand. She studied in the field of Fermentation technology at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology from December 2022 to September 2024 with the thesis entitled "Isolation and Identification of Microorganisms for Xylitol Production from Lignocellulosic Hydrolysates".