MODEL OF USING *VIETNAMOSASA PUSILLA* AS STARTING MATERIAL FOR THE PRODUCTION OF ETHANOL AND XYLITOL

SUWANAN WONGLEANG

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
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has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Biotechnology of Naresuan University

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ABSTRACT

Lignocellulosic biomass can be used as a renewable and sustainable energy source to help reduce the consequences of global warming. In the new energy age, the bioconversion of lignocellulosic biomass into green and clean energy displays remarkable potential and makes efficient use of waste. Bioethanol is a biofuel that can reduced reliance on fossil fuels while minimizing carbon emissions and increasing energy efficiency. Various lignocellulosic materials and weed biomass species have been selected as potential alternative energy sources. *Vietnamosasa pusilla*, a weed belonging to the Poaceae family, contains more than 40% glucan. However, research on the applications of this material is limited. Thus, we aimed to achieve maximum fermentable glucose recovery and bioethanol production from *V. pusilla*. Two pretreatment reagents were applied, H_3PO_4 (phosphoric acid) and NaOH (sodium hydroxide), to disrupt the recalcitrant structure of the lignocellulosic biomass.

First section, *V. pusilla* feedstocks were treated with varying concentrations (70%, 75%, 80%, and 85%) of H_3PO_4 and then subjected to enzymatic hydrolysis. The results indicated that after pretreatment with different concentrations of H_3PO_4, the glucose recovery and digestibility at each concentration were markedly enhanced. Moreover, 87.5% of cellulosic ethanol was obtained from *V. pusilla* biomass hydrolysate medium without detoxification.

In the next section, the *V. pusilla* feedstocks were again pretreated by NaOH pretreatment-assisted autoclaving. The *V. pusilla* enzymatic hydrolysate was used as a substrate for bioethanol and xylitol synthesis. After treating the feedstock with varying concentrations (1%, 2%, 3%, and 4%) of NaOH at different temperatures (110 °C, 120 °C, and 130 °C), the glucose and xylose recovery yields were substantially higher than those of the untreated material. The hydrolysate generated by enzymatic hydrolysis was fermented into bioethanol using *Saccharomyces cerevisiae* TISTR 5339. The liquid byproduct of ethanol production was utilized by *Candida tropicalis* TISTR 5171 to generate xylitol.

Overall, our findings reveal that *V. pusilla* biomass can be introduced into sugar-based biorefineries to produce biofuels and other valuable chemicals.

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Suwanan Wongleang

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ABBREVIATIONS

ASL	=	Acid soluble lignin
AIL	=	Acid insoluble lignin
BH	=	Biomass hydrolysate
CrI	=	Crystallinity index
FESEM	=	Field Emission Scanning Electron Microscope
GR	=	Glucose recovery
HEG	=	Hydrolysis efficiency of glucose
HEX	=	Hydrolysis efficiency of xylose
HPLC	=	High Performance Liquid Chromatography
NREL	=	National Renewable Energy Laboratory
ODW	=	Oven dry weight
rpm	=	round per minute
XR	=	Xylose recovery
XRD	=	X-ray diffraction

CHAPTER I

OVERALL INTRODUCTION

Background

The depletion of nonrenewable fossil fuel reserves is impending, leading to a severe global energy crisis (Khan et al., 2021; Tusher et al., 2022). Numerous countries have formulated plans to reduce greenhouse gas emissions by transitioning to clean technology sources, such as ethanol. Renewable energy derived from lignocellulosic biomass holds the potential to replace the reliance on fossil fuel-based generation (Casau et al., 2022; Khan et al., 2021; Shukla et al., 2023; Tusher et al., 2022; Zhang et al., 2022).

Lignocellulosic biomass is abundant, cost-effective, and may form part of an environmental friendly conversion process (Sun et al., 2022). Extensive research has identified lignocellulose as an alternative energy source because of its high cellulose content, which makes it a suitable bioethanol production substrate (Lee et al., 2022; Messaoudi et al., 2022; Sun et al., 2022; Wongleang et al., 2023b).

Vietnamosasa pusilla, commonly known as Pai Pek (local Thai name), belongs to the Poaceae family and is a drought-resistant weed. *V. pusilla* has been classified as a regionally controlled weed, indicating its status as a risk group for a specific region's primary crops and the environment (Pagad, 2016; Thuy et al., 2021). The major advantage of weed biomass is its rapid development despite harsh environmental conditions and minimal cultivation requirements (Obeng et al., 2019). Utilizing this material not only reduces environmental issues but also increases its value.

Nevertheless, the intricate and recalcitrant structure of lignocellulosic biomass, comprising hemicellulose, lignin, and cellulose, exerts a significant impact on enzymatic saccharification, thereby reducing the bioconversion yield (Shukla et al., 2023; Zhang et al., 2022; Zhao et al., 2022). Consequently, pretreatment is required to disintegrate the lignin structure and making it more accessible to the enzymatic hydrolysis (Shukla et al., 2023; Zhao et al., 2022).

Among the various pretreatment methods, the fractionation of lignocellulosic biomass using acid or alkaline reagents has received considerable attention due to its effectiveness and ecological advantages (Zhang et al., 2023b). Two pretreatment reagents were applied, H_3PO_4 and NaOH, to disrupt the recalcitrant structure of the lignocellulosic biomass. Pretreatment with H_3PO_4 has demonstrated greater effectiveness under mild reaction conditions (Satari et al., 2019). The pretreated samples exhibited high hydrolysis efficiency and minimal inhibitor generation. Additionally, the residual pretreated samples did not prevent enzymatic saccharification or fermentation (Satari et al., 2019). Several studies have highlighted the ability of H_3PO_4 pretreatment of lignocellulosic biomass resources to enhance ethanol yield (Satari et al., 2019). Moreover, H_3PO_4 is inexpensive, less toxic and corrosive compared to other acids. Additionally, phosphoric salts produced through neutralization at the end of the pretreatment process can be utilized as fertilizers, fermentation buffers, and for microorganism nutrition (Premjet et al., 2016; Satari et al., 2019).

Alkaline pretreatment has gained widespread acceptance due to its simplicity, non-corrosiveness, absence of pollutants, and strong pretreatment effectiveness under mild reaction conditions, leading to fewer inhibitory compounds (An et al., 2021). This method disrupts a complex lignin–hemicellulose network, enhancing lignin removal and increasing the surface area and porosity of the biomass. Consequently, enzymatic hydrolysis is enhanced, preserving a higher fraction of carbohydrates (Olatunji et al., 2022; Shukla et al., 2023; Zhao et al., 2022). The effectiveness of the NaOH pretreatment can be improved by integrating a heating method that involves subjecting the material to high temperatures and pressures. NaOH-assisted thermal pretreatment is considered a highly efficient method for breaking down the recalcitrant structure of lignocellulose while minimally impacting cellulose and hemicellulose. This process effectively removes a large amount of lignin, reduces cellulose crystallinity, and enhances the specific surface area of cellulose for enzymatic saccharification (Obeng et al., 2021; Shukla et al., 2023; Zhao et al., 2022).

This study aimed to determine the effects of two pretreatment reagents on *V. pusilla* feedstock. The *V. pusilla* hydrolysate was used as a substrate for bioethanol production, while by-product from ethanol fermentation was used for xylitol

production. Our findings may help optimize the efficiency of cellulosic ethanol and xylitol production using plant-based biomass.

Research Objectives

The major objective of this research is to enhance the potential of *V. pusilla* as feedstocks for production of various biochemicals such as bioethanol, xylitol by developing an optimal pretreatment condition for their conversion to fermentable sugar.

To evaluate the effect of H_3PO_4 pretreatment on the chemical composition, structural changes, and monomer sugar production.

To evaluate the effect of NaOH-assisted thermal pretreatment on the chemical composition, structural changes, and monomer sugar production.

Research Hypothesis

Optimal H_3PO_4 pretreatment conditions can disrupt the recalcitrant structure of *V. pusilla* biomass to improve the efficiency of enzymatic hydrolysis and sugar recovery yields for bioethanol production.

Optimal NaOH-assisted thermal pretreatment conditions can disrupt the recalcitrant structure of *V. pusilla* biomass to improve the efficiency of enzymatic hydrolysis, sugar recovery yields for bioethanol production.

Research Significance

This research will provide an effective and efficient approach for managing the invasive weed *V. pusilla* by converting it into valuable bioethanol and xylitol. The outcome of this study will be very beneficial in the energy sector. In the current energy research landscape, there is a notable shift towards utilizing non-edible lignocellulosic biomass for bioethanol production. The utilization of bioethanol as an alternative fuel holds promise as a sustainable and eco-friendly alternative to traditional fossil fuels.

CHAPTER II

LITERATURE REVIEW

Global Energy Consumption

Energy plays a crucial role in influencing socio-economic growth, with fossil fuels maintaining their dominance as the primary energy source. As highlighted in the Global Status Report (GSR) on energy, non-renewable fossil fuels such as petroleum, coal, and natural gases constitute 78.4% of total energy consumption. In contrast, renewable alternative energy, including hydropower, wind, solar, and biomass collectively represent a more modest share of 19% (Aggarwal et al., 2022; Rumyantseva et al., 2019).

The Environmental Impact and Constraints of Fossil Fuel Utilization

Fossil fuels have a strong economic appeal due to their low cost. However, a careful evaluation shows that using them poses a number of challenges. Firstly, the combustion of fossil fuels is a major contributor to global warming and other forms of pollution (Jeevan Kumar et al., 2020; Straathof et al., 2019; Zhao et al., 2022).

The limited and non-renewable nature of fossil fuels is a cause of concern for their long-term sustainability. This limitation has resulted in severe energy security issues, with reserves concentrated in politically unstable regions of the world, volatile price fluctuations, and environmental concerns related to their production. These challenges significantly impact the chemical industry, which relies heavily on these fuels to produce diverse range of chemicals. Moreover, it limits the potential of fossil fuels to meet future energy and chemical demands (Aggarwal et al., 2022; Asomaning et al., 2018; Tusher et al., 2022). Therefore, it is essential to explore alternative energy sources to reduce our reliance on fossil fuels and address both environmental and industrial concerns.

Sustainable and Alternative Energy Source

The energy demand is increasing daily due to population growth, economy expansion, and industrialization (Aggarwal et al., 2022). This surge in global energy

demand is projected to increase by 50% by 2025, leading to a worldwide energy shortage. In the past, people used fossil fuels without considering the social consequences, such as environmental and health issues. However, over the last four decades, both the public and scientific communities have become more aware of the harmful effects of excessive fossil fuel use on the environment and global warming. The socioeconomic impact of this process includes environmental effects due to pollutant release, health impact on workers and nearby residents, economic growth, and energy security, which were assessed (Aggarwal et al., 2022; Rass-Hansen et al., 2007).

Concerns about energy security, fossil resource depletion, and global warming have prompted scientists to search for alternative, sustainable, and renewable energy systems (Aggarwal et al., 2022; Broda et al., 2022). To achieve sustainable development, countries worldwide have implemented policies aimed at increasing the cost-effective biomass utilization to meet future energy demands. These policies also aim to achieve the Kyoto Protocol's CO_2 reduction targets and reduce reliance on fossil fuel supply (Aggarwal et al., 2022).

Renewable feedstocks that can be used to produce fuels, chemicals, and other products have replaced fossil resources. The bioconversion of lignocellulosic materials to green and clean energy efficiently utilizes waste and demonstrates significant application potential in the new energy era (Kumar et al., 2020; Straathof et al., 2019; Zhao et al., 2022). The projected benefits are so substantial that economists, researchers, and state policymakers have invented the term "bio-based economy" to describe an economy based on bio-based products. It is also referred to as a "circular bioeconomy" due to its renewable nature (Venkata Mohan et al., 2016).

Bioethanol

Bioethanol, or ethyl alcohol, is the most commonly researched and manufactured biofuel, accounting for over 90% of the biofuel market. It offers several benefits, including the potential to reduce CO_2 emissions by up to 80%, emit fewer pollutants than traditional petroleum-based fuels, improve energy efficiency, and reduce dependence on fossil fuels. Furthermore, the carbon dioxide produced during bioethanol burning is similar to the CO_2 used by plants for growth and metabolism, which means that it does not contribute to greenhouse gas emissions. This makes it a

promising option for creating a cleaner environment (Aggarwal et al., 2022; Ayodele et al., 2019; Chen et al., 2021; Satari et al., 2019).

Bioethanol is made primarily from sugar, starch, and cellulose feedstock. It can be used as a sole fuel (E85) or as an octane enhancer in a blend with gasoline (known as E10 or gasohol). E10 is a mix of 10% anhydrous ethanol and 90% gasoline that can be used in most modern vehicles and light trucks without any engine or fuel system modifications. On the other hand, E85, which is a blend of 85% ethanol and 15% gasoline, is primarily used in flexible-fuel vehicles (FFVs) and variable-fuel vehicles (VFVs) (Aggarwal et al., 2022; El Hage et al., 2022; Toor et al., 2020).

Ethanol is a high-quality fuel that is particularly suitable for racing. It burns at a lower temperature than gasoline, requiring less radiator cooling power. Using ethanol fuel blends such as E85 can reduce total greenhouse gas emissions by up to 30–36% while decreasing fossil energy use by 42–48%. Furthermore, ethanol-blended fuel (E10) can reduce greenhouse gas emissions by 2.4–2.9% and fossil energy consumption by 3.3–3.9% (Aggarwal et al., 2022; Hu et al., 2004; Toor et al., 2020).

Bioethanol Trends

The demand for ethanol was rising before COVID-19. The largest consumers and producers of ethanol are the United States, Brazil and European Union, which account for around 86% of global production in 2022 (Figure 1) (Sönnichsen, 2023).

Thailand ranks 7th in the world for ethanol production and consumers. Thailand uses molasses, cassava, and sugarcane as primary inputs for ethanol production; these three accounted for 58%, 38%, and 4% of all ethanol produced in the country (Figure 2). Government support for the biofuel sector and promotion of gasohol mixes containing a higher proportion of ethanol (E20 and E85 gasohol) will help the growth of the Thailand ethanol industry. However, the COVID-19 pandemic disrupted the ethanol market, reducing travel and cutting car use. Demand for industrial-grade ethanol surged for hand sanitizers and pure alcohol (Research, 2021; Sönnichsen, 2023).

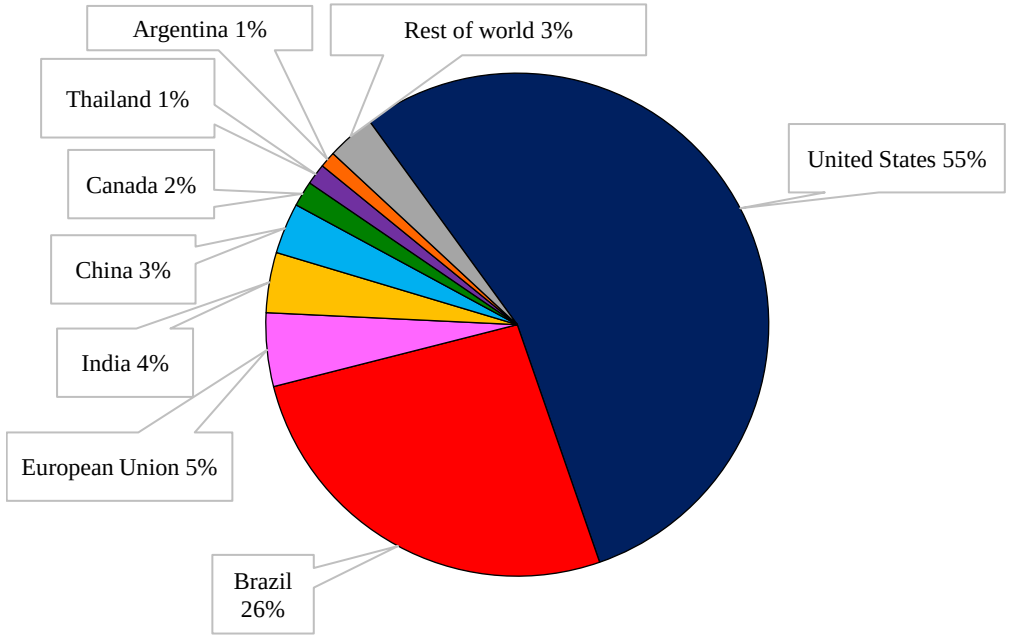

Figure 1 Global ethanol production by country in 2022 (millions of gallons)

Source: Sönnichsen, 2023

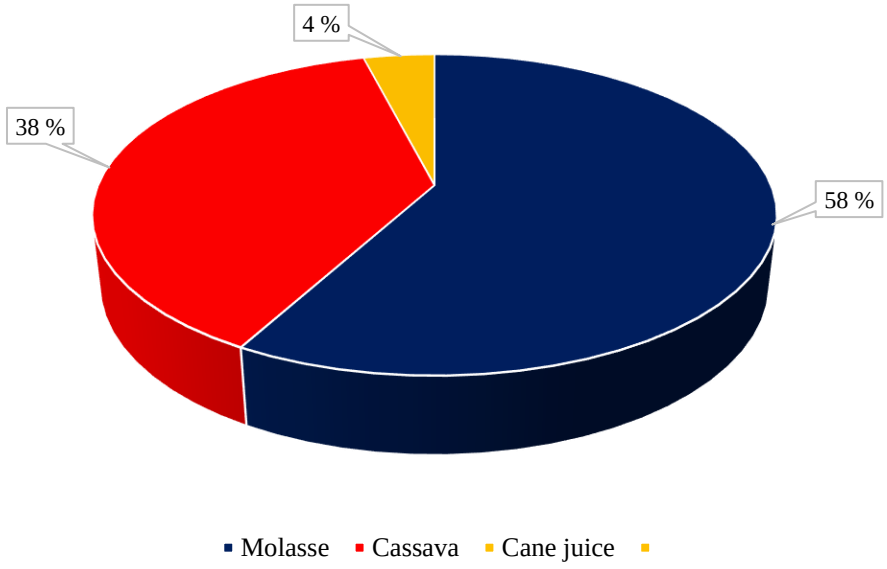

Figure 2 Ethanol production in Thailand by raw materials

Source: Research, 2021

In 2020, the ethanol market was disrupted due to the COVID-19 pandemic and the severe drought which led to a sharp increase in input costs for ethanol producers. The margins for ethanol producers using molasses have narrowed to their slimmest in a decade due to the price rises of inputs. The cost of molasses has reached a 10-year high of THB 5.8 per kilogram, and the drought has affected the supplies. Consequently, production costs have increased, leading to volatile price fluctuations that affect energy security.

Demand for ethanol is expected to increase by 2.4-3.6% annually between 2021-2023, resulting in a total daily consumption of 4.2-4.5 million liters. This growth is driven by government policies and initiatives promoting biofuels.

Bioethanol Production from Lignocellulosic Biomass

Bioethanol is a fuel derived from biomass and is generally categorized into three main groups. The first category, known as 1G bioethanol, is derived from sugar-based plants such as sugarcane and sugar beet, starch-rich crops like maize and wheat grains, and specific root and tuber crops. However, this bioethanol is associated with concerns about food security and land use (Aggarwal et al., 2022; Premjet et al., 2013).

In contrast, the second category, also known as 2G bioethanol or cellulosic bioethanol, is produced from cellulosic materials, including crop residues like rice straw and maize stover, and woody materials (Table 1). It can be produced by fermentable sugar from the hydrolysis of lignocellulosic biomass. The advantage of 2G bioethanol is that it presents no risk to food security, has a low and constant price, and requires no additional land. Cellulosic biomass has been found to have better potential for bioethanol generation than starch and sugar crops due to its higher yield, availability, and established processing methods. Moreover, its cost-effectiveness and environmental friendliness align with sustainability goals and circumvent challenges associated with traditional feedstocks (Aggarwal et al., 2022; Bušić et al., 2018).

It is important to note that the quality and cost of bioethanol are heavily influenced by the production pathways used. Creating bioethanol involves multiple stages, and each stage can produce varying quality and cost of ethanol. When using lignocellulosic material for bioethanol production, four essential processes are

involved: pretreatment of the biomass, enzymatic saccharification, fermentation, and distillation (Aggarwal et al., 2022).

Table 1 Bioethanol production from lignocellulosic biomass

Materials	Yeast	Ethanol yield* (g/g)	Reference
Elephant grass	*Saccharomyces cerevisiae* PE-02 and *Meyerozyma caribbica* CHAP-096	0.42	(Vargas et al., 2023)
Corn fiber	*S. cerevisiae* LF1 and 6M-15	0.47	(Li et al., 2023b)
Corn stover	*S. cerevisiae XH7*	0.44	(Sun et al., 2022)
Giant reed	*S. cerevisiae*	0.13	(Shafaei et al., 2023)
Poplar	Dried yeast	0.44	(Li et al., 2023a)
Rice straw	*S. cerevisiae* NCIM 3280 and *Zymomonas mobilis* MTCC 2427	0.36 and 0.20	(Kumar et al., 2023)
Cassava	*Clostridium thermocellum ATCC 31,924*	0.32	(Papathoti et al., 2022)
Palm fronds	*S. cerevisiae*	0.21	(Messaoudi et al., 2022)
Eucalyptus chips	*S. cerevisiae*	0.14	(Messaoudi et al., 2022)

Note: *Ethanol yield is given as grams of ethanol per gram of glucose consumed.

Lignocellulosic Materials

Lignocellulosic biomass is the world's largest source of natural carbohydrates. It is plentiful among diverse materials, with a yearly production of 200,109 tons annually. Recently, lignocellulosic materials have demonstrated their potential as feedstocks for bioethanol production because they are an abundant, sustainable, and inexpensive organic feedstock for producing renewable energy (Aggarwal et al., 2022; Chen et al., 2021; Kumar & Sharma, 2017; Kumar et al., 2020; Satari et al., 2019).

Lignocellulosic biomass is made up of three majority polymers, cellulose (35–50%), hemicellulose (20–35%), and lignin (5–30%), as well as a small amount of ash and extractives (Yu et al., 2017).

Cellulose

Cellulose, or the glucose polymer, is a structural part of lignocellulosic biomass linked to reduced or non-reduced ends via a β-(1–4) glycosidic bond. The problematic and recalcitrant nature of cellulose is due to its high degree of crystallinity in structure, higher polymerization of approximately 10,000 units, and complex network of inter- and intra-molecularly hydrogen-linked hydroxyl groups (Aggarwal et al., 2022; Satari et al., 2019; Shukla et al., 2023).

Hemicellulose

Hemicellulose is an amorphous branched polymer. It is made up of pentose (xylose, arabinose) and hexoses (mannose, glucose, galactose) with at least 500-3000 sugar monomers connected by ester bonds (Satari et al., 2019; Shukla et al., 2023; Zhang et al., 2011). Hemicellulose structures have a lower degree of polymerization than cellulose. Therefore, the hemicellulose fraction has little effect on the recalcitrant nature of lignocellulosic biomass (Aggarwal et al., 2022; Satari et al., 2019). Hemicellulose imparts the overall biomass structure stiffness by acting as a binder between the cellulosic and lignin sections. Both cellulose and hemicellulose contain reducing sugars, making them an important source of many economically viable compounds (Aggarwal et al., 2022; Quereshi et al., 2019; Shukla et al., 2023).

Lignin

Lignin is a complex polymer that provides structural support to plants and forms a network with cellulose microfibrils. It is composed of three main monolignols: coniferyl alcohol (G-units), paracoumaryl alcohol (H-units), and sinapyl alcohol (S-units). These monolignols are joined by C–O–C and C–C bonds (Aggarwal et al., 2022; Shukla et al., 2023; Zhang et al., 2011). However, lignin is tightly bound to cellulose or hemicelluloses, which makes it difficult for enzymes like cellulases or hemicellulases to break them down. Converting lignin into hydrolysate can produce valuable compounds and economically beneficial chemicals (Aggarwal et al., 2022; Satari et al., 2019).

According to Table 2, the percentage mix of the three significant components in biomass may vary significantly depending on its source. The chemical composition of lignocellulosic materials also varies in terms of the amount and type of carbohydrates and non-cellulosic carbohydrates present, as well as the presence of proteins or phenolic chemicals (Aggarwal et al., 2022; Zhang et al., 2011).

Table 2 Chemical composition of various lignocellulosic feedstocks

Biomass	Cellulose (%dw)	Hemicellulose (%dw)	Lignin (%dw)	References
Cassava	32.4 ± 0.6	20.1 ± 0.4	14.6 ± 0.2	(Papathoti et al., 2022)
Corn fiber	26.3 ± 1.3	37.6 ± 1.0	2.1 ± 0.1	(Li et al., 2023b)
Corn straw	34.3 ± 0.5	17.6 ± 0.4	21.9 ± 0.2	(Jin et al., 2023)
Corn stover	31.2 ± 0.6	21.4 ± 0.3	18.9 ± 0.7	(Sun et al., 2022)
Sugarcane bagasse	41.2	20.2	25.2	(Zhang et al., 2023)
Jute biomass (bark)	53.9 ± 0.8	6.8 ± 0.1	16.3 ± 0.1	(Wongleang et al., 2023a)
Elephant grass	35.7 ± 3.0	15.3 ± 2.7	18.0 ± 1.0	(Vargas et al., 2023)
Arecanut husk	43.3 ± 0.4	29.2 ± 0.3	12.6 ± 0.1	(Vardhan et al., 2023)
Poplar	43.1 ± 0.9	16.0 ± 0.4	23.6 ± 0.0	(Li et al., 2023a)
Quinoa straw	31.0 ± 0.5	20.8 ± 0.6	20.0 ± 0.6	(Jin et al., 2023)
Palm fronds	39.9 ± 0.8	28.1 ± 0.5	22.5 ± 0.5	(Messaoudi et al., 2022)
Eucalyptus chips	43.2 ± 0.4	30.5 ± 2.9	19.2 ± 2.2	(Messaoudi et al., 2022)
Almond shells	41.4 ± 2.8	28.5 ± 0.2	13.7 ± 3.2	(Messaoudi et al., 2022)

Weed Biomass

The term weed biomass is used to describe plants that grow on agricultural land but are not wanted for agriculture. These plants are considered agricultural pests because they compete with crops for essential resources, including nutrients, minerals, water, and sunlight (Chandel & Singh, 2011). Weeds biomass can be a profitable and valuable resource for potential biofuel feedstocks due to their rapid reproduction, high cellulose content, and low lignin content (Aggarwal et al., 2022). Multiple weeds, including *Sida acuta*, *Leucaena leucocep hala*, *Achyranthes aspera* (Premjet et al., 2016; Premjet et al., 2013), *Miscanthus* × *giganteus* (Ji et al., 2015), *Arundo donax*, *Medicago sativa*, and *Pennisetum giganteum* (Zhang et al., 2020b) have been discovered as potential sources of alternative energy due to their high cellulose content. Also, most weeds in nature survive on marginal land and produce a high amount of biomass, even though they have limited access to water and nutrients (Premjet et al., 2016).

Vietnamosasa pusilla, or "Pai Pek," is a weed belonging to the Poaceae family (Figure 3). It is usually found in semi-deciduous forests in regions with a climate that varies seasonally and on plateaus. This material has the advantage of being able to tolerate drought conditions and grow throughout the year. As a result, this raw material for the industry remains stable. In addition, its comparatively high polysaccharide content can facilitate the production of biofuels and bio-based compounds. *V. pusilla* has been declared a "regionally controlled weed," a group of noxious weeds that are risky for a region's main crop or environment and are likely to spread within that region or to another (Jin et al., 2021; Pagad, 2016; Thuy et al., 2021). Utilizing this material not only reduces environmental issues but also increases its value. However, research on this material's application has been limited.

Figure 3 ***Vietnamosasa pusilla***

Lignin Carbohydrate Complexes

Converting lignocellulose to bioethanol is substantially more complicated than sugar or starch conversion. It is challenging to convert lignocellulosic biomass to biofuels due to its complicated structure, making it resistant to enzymatic hydrolysis (Aggarwal et al., 2022). In order to effectively utilize the material, due to the recalcitrance structure of the biomass, it is necessary to modify the material's structure so that the carbohydrate fraction can be broken down into monomer sugars (Satari et al., 2019). Therefore, the pretreatment step is necessary to remove lignocellulosic biomass's natural core, which is made up of cellulose and hemicellulose encased in a matrix of a very recalcitrant substance called lignin by fractionating the biomass into its components. After that, the carbohydrate polymers of cellulose or hemicellulose are hydrolyzed to their respective monomers, such as hexose and pentose sugars, which are then transformed into bioethanol in the subsequent fermentation stage. The process of pretreatment plays a crucial role in making cellulose and hemicellulose more accessible

to hydrolyzing enzymes, which are then fermented to produce biofuels (Aggarwal et al., 2022; Kumar et al., 2020; Shukla et al., 2023; Zhao et al., 2022).

Pretreatment of Lignocellulosic Biomass

Pretreatment is essential in converting biomass into high-value products like sugars and biofuels. Different techniques are used to overcome the recalcitrance of lignocellulosic biomass and accelerate its decomposition into individual units, such as cellulose, hemicellulose, and lignin. Various strategies have been developed, classified as physical, chemical, physicochemical, and biological (Aggarwal et al., 2022; Shukla et al., 2023).

Physical Pretreatment

The physical pretreatment method can enhance available surface area and pore size and lower cellulose crystallinity and grade of polymerization. Moreover, it reduced the amount of chemicals used during the process and minimized the chemical waste produced after the treatment (Moset et al., 2018; Shukla et al., 2023).

Most processes have used mechanical methods, such as gridding and chipping, to lower the particle sizes of biomass feedstocks. Various physical processes like milling, including two-roll milling, ball milling, hammer milling, vibro-energy milling, and colloid milling, can be utilized as a pretreatment. Additionally, irradiation techniques such as gamma rays, microwaves, electron beams, and ultrasounds have been employed to improve the enzymatic degradation or biodegradability of lignocellulosic feedstocks (Aggarwal et al., 2022; Shukla et al., 2023).

Physical processes, in general, contribute to higher operational costs because of their high energy utilization. Furthermore, physical procedures remove less lignin than chemical pretreatment from the cell wall composition. Enhancing physical treatment performance is often combined with other types of pretreatment, as highlighted in recent research (Aggarwal et al., 2022; Shukla et al., 2023).

Chemical Pretreatment

Chemical pretreatment has long been the most common process for saccharification of cellulose. This method usually uses a variety of chemical reagents such as acids, alkalis, organic solvents, ozone, and peroxide. Concentrated acids like

sulfuric acid and hydrochloric acid are particularly helpful in improving the enzymatic hydrolysis of fermentable sugars. Chemical pretreatment has been recognized as an efficient way to treat a wide range of biomass feedstocks because it can degrade hemicellulose and lignin, thus enhancing cellulose hydrolysis. However, these chemicals are highly toxic and corrosive. In addition, it requires specialized reactors, which can increase costs (Peral, 2016; Satari et al., 2019; Shukla et al., 2023).

The chemical pretreatment by H_3PO_4 is more engaging because several lignocellulosic materials have been successfully pretreated using concentrated H3PO4 (85%) at mild reaction conditions (50-60 °C). Hydrolysis efficiency for pretreated samples is very high. For instance, after 72 hours of hydrolysis, the digestibility of glucose was more excellent than 90%, with less inhibitor generation. Furthermore, the remaining pretreated sample did not prevent the enzymatic hydrolysis or fermentation processes. Moreover, other studies have observed H_3PO_4 pretreatment to increase ethanol yield. In addition, H_3PO_4 is less poisonous and corrosive than other acids and inexpensive compared to other mineral acids. In addition, phosphoric salts generated by the neutralization at the end of the pretreatment process can be utilized as fertilizers, fermentation buffers, and microorganism nutrition (Premjet et al., 2016; Satari et al., 2019; Zhang et al., 2010).

The procedure of alkaline pretreatment involves adding diluted bases to biomass mixtures, like sodium hydroxides, potassium hydroxides or anhydrous, calcium, and ammonia, to cleave the linkages present between the lignin and other compounds (Aggarwal et al., 2022). The processes speed up the delignification step by breaking the lignin structure and eliminating the lignin, which reduces the plant cell's mechanical strength. As a result, enzymes have better access to cellulose components of the cell, and sugar liberated from processed material during digestion is better (Aggarwal et al., 2022; Ethaib et al., 2020). NaOH pretreatment is a commonly used alkaline pretreatment method that effectively breaks down lignin. This is achieved by solubilizing the bonds between lignin and carbohydrates, increasing the surface area of cellulose, and retaining the sugar content (Shukla et al., 2023; Zhao et al., 2022). As a result, enzymatic hydrolysis is enhanced, and carbohydrates are better preserved (Olatunji et al., 2022; Shukla et al., 2023; Zhao et al., 2022).

Physicochemical Pretreatment

Physicochemical processes use a combination of physical equipment and chemical chemicals to perform preparation. These procedures can be far more effective than only using physical mechanisms. One of the most studied physicochemical methods is steam explosion. In a steam explosion, pressure is abruptly lowered, causing the materials to decompress explosively (Aggarwal et al., 2022). This approach has used higher pressure and higher temperature for a short period (Aggarwal et al., 2022; Baêta et al., 2017). High-pressure water can enter the biomass, hydrate cellulose, and remove hemicellulose and some lignin. This technique dissolves around 40–60% of total biomass, 4–22% of cellulose, 35–60% of lignin, and all hemicellulose (Kumar et al., 2011). Although this treatment has several advantages, including quick treatment duration, no use of chemicals, and minimal energy consumption, there are concerns about lignin removal, xylan breakdown into hemicellulose, and the risk of producing inhibitory compounds during high-temperature processing (Aggarwal et al., 2022).

Steam explosion pretreatment can be done with the addition of various chemicals, such as organosolvents, NaOH, and H_2SO_4, to encourage hemicellulose degradation, improve lignin solubilization, and reduce inhibitor synthesis if reduced temperatures are used, resulting in improved enzyme accessibility to cellulose in subsequent processing (Aggarwal et al., 2022; Guerrero et al., 2017; Martino et al., 2017; Obeng et al., 2021; Shukla et al., 2023; Zhao et al., 2022).

Table 3 Improvement in lignin removal and hydrolysis efficiency yield from different lignocelluloses pretreated with physicochemical methods

Feedstock	Conditions	Lignin removal	HE (%)	Reference
Sorghum	2% NaOH at 121 °C for 60 min	75%	-	Vancov and McIntosh, 2011
Imperata cylindrica	20 mg/mL NaOH at 105 °C for	74%	-	Haque et al., 2016
Miscanthus floridulus	6% NaOH at 35 °C for 3 h	11.8%	-	Fu et al., 2018
Durian peel	2% NaOH at 110 °C for 60 min	74-77%	85-90	Obeng et al., 2021
Sida acuta	3% NaOH at 60 °C for 9 h	60%	67	Premjet et al., 2018
Bamboo	85% H_3PO_4 at 50 °C for 60 min	33%	88	Sathitsuksanoh et al., 2010
pineapple leaf	85% H_3PO_4 at 50 °C for 45 min	30-40%	83-87	Mund et al., 2021
Corn stover	85% H_3PO_4 at 50 °C for 3 h	33%	-	Wang et al., 2016
Hibiscus sabdariffa	70-75% H_3PO_4 at 60 °C for	40-55%	91.7-95.4	Premjet et al., 2018b
Durio zibethinus	75% H_3PO_4 at 60 °C for 60 min	60%	90	Obeng et al., 2018

Biological Pretreatment

Microorganisms can be used to process biomass and improve the rate of enzymatic degradation or fermentation. Various bacteria strains, including, *Actinomycetes*, *Candida*, *Bacillus*, and *Streptomyces*, as well as certain well-known fungal species, for example—*Ceriporia lancerata* and *Cyathus stercolerus*, are used to breakdown lignocellulosic biomass (Pandey et al., 2014). Biological pretreatments consume less energy and can be carried out in a controlled setting. However, most of these techniques require extended incubation times and are not as efficient, which limits their industrial use (Zabed et al., 2019; Zhao et al., 2022).

Biorefinery of Bioethanol Production

The depletion of fossil sources and increasing energy needs have driven the call for alternative and clean energy. Using lignocellulosic biomass for large-scale biorefinery applications has become more accessible, cost-effective, and environmentally beneficial (Aggarwal et al., 2022).

Biorefinery is a broad term that encompasses diverse sectors like chemistry, transport, energy, and agriculture. The International Energy Agency (IEA)has highlighted biorefining as a sustainable way to convert biomass into various bio-based products and biofuels, effectively using available feedstock for energy production. According to another definition, the biorefinery is defined as "a facility for the synergetic conversion of biomass into multiple commercial biobased products (food and feed ingredients, chemicals, materials, minerals, CO_2) and bioenergy (fuels, power, heat)" (Aggarwal et al., 2022; ETIP, 2017).

Bioethanol production from lignocellulosic biomass indicated that pretreatment's operating cost be expressed as a percentage of the entire operating cost. Using waste substrate or low-cost materials to generate power makes the process more cost-effective (Aggarwal et al., 2022).

Value-Added Chemicals

Bioethanol is used in the transport sector; on the other hand, bioethanol is used as a substrate for industrial applications, stating that bioethanol may be used to produce acetaldehyde, ethylene, butadiene, and acetic acid, among other things (Rass-Hansen et al., 2007). Furthermore, the use of ethanol as a carbon source for the production of L-

glutamic acid in *Brevibacterium* sp., *Corynebacterium* sp., and *Pseudomonas*, *Alcaligenes*, and *Bacillus* strains (Aggarwal et al., 2022; Oki et al., 1968). Additionally, ethanol as a source of carbon for the manufacture of L-lysine using *C. glutamicum*, *Brevibacterium ammoniagenes*, *Bacillus megaterium*, *Arthrobacter paraffineus*, and *Nocardia* strains (Aggarwal et al., 2022). Moreover, vinegar is manufactured with bakers' yeast, initially converting sugar to alcohol, followed by a second fermentation process in which the alcohol is transformed into acetic acid by *Acetobacter* species (Solieri & Giudici, 2008).

CHAPTER III

CELLULOSIC ETHANOL PRODUCTION FROM WEED BIOMASS HYDROLYSATE OF *VIETNAMOSASA PUSILLA*

Introduction

Energy is a vital aspect that determines the socioeconomic development of a nation, especially fossil fuels, which are the principal source of energy. However, combustion of fossil fuels is one of the leading contributors to global warming ant other forms of pollution. Mitigating greenhouse gases and diminishing reliance on fossil fuels is the impetus for the pursuit of alternative energy sources. To achieve sustainable development goals, fossil resources should be substituted with biomass feedstocks that can produce various products, including biofuels, green chemicals, and product lines for other industries (Kumar et al., 2020; Straathof et al., 2019; Zhao et al., 2022). Utilizing lignocellulosic biomass as a sustainable and renewable source of energy could mitigate the consequences of global warming (Patel & Shah, 2021). In the new energy age, the bioconversion of lignocellulosic biomass to green and clean energy has tremendous application potential and makes efficient use of waste (Kumar et al., 2020; Straathof et al., 2019; Zhao et al., 2022). Among biofuels, bioethanol has been the most researched and manufactured in factories. Bioethanol can reduce carbon emissions, improve energy efficiency, and make a country less dependent on fossil fuels (Ayodele et al., 2020; Chen et al., 2021; Satari et al., 2019). Several lignocellulosic materials have recently proven their potential as cellulosic ethanol feedstocks owing to their abundance, sustainability, renewable nature, and low cost (Chen et al., 2021; Kumar & Sharma, 2017; Kumar et al., 2020; Satari et al., 2019). Furthermore, several weed biomass species, including *Sida acuta*, *Leucaena leucocephala*, *Achyranthes aspera* (Premjet et al., 2016; Premjet et al., 2013), *Miscanthus* × *giganteus* (Ji et al., 2015), *Arundo donax*, *Medicago sativa*, and *Pennisetum giganteum* (Zhang et al., 2020b), have been recognized as possible sources of alternative energy because of their high cellulose content. In addition, most weeds in nature survive on marginal lands and produce a high

amount of biomass, despite having limited access to water and nutrition (Premjet et al., 2016).

Vietnamosasa pusilla or Pai Pek (Thai local name), is a weed that belonging to the Poaceae family. It is usually found on plateaus and in semi-deciduous forests in regions with climates that vary seasonally. This weed has the advantage of being able to tolerate drought conditions and grows throughout the year. *V. pusilla* has been declared a "regionally controlled weed", i.e., it is a group of noxious weeds that are risky for a region's main crop or environment and are likely to spread within that region or to another (Jin et al., 2021; Pagad, 2016; Thuy et al., 2021). Utilizing this weed's biomass reduces environmental issues but also increases its value. However, research on the applications of this material is limited. One of the most significant obstacles in employing lignocellulosic biomass for biofuel production is the complicated and naturally robust structure of plant cell walls that hinders the hydrolysis of cellulose (Chandel et al., 2018; Mankar et al., 2021; Zoghlami & Paës, 2019). Consequently, several pretreatment procedures have been used to disintegrate the structure of the lignocellulosic biomass, causing the removal of lignin and hemicellulose, which are then transformed into monomer sugars via enzyme hydrolysis. The sugar is then fermented by microbes to produce bioethanol (Chandel et al., 2018; Patel & Shah, 2021).

Chemical pretreatment with phosphoric acid (H_3PO_4) has been shown to be more effective as several lignocellulosic materials have been successfully pretreated using concentrated H_3PO_4 under mild reaction conditions (Satari et al., 2019). The hydrolysis efficiency of the pretreated samples was very high, with less inhibitor generation. Furthermore, the remaining pretreated samples did not prevent enzymatic hydrolysis or fermentation (Satari et al., 2019). Several studies have demonstrated that H_3PO_4 pretreatment of lignocellulosic biomass resources, including *Tripsacum dactyloides*, *Populus tremula*, and oil palm empty fruit bunches can enhance ethanol yield (Satari et al., 2019). In addition, H_3PO_4 is less poisonous and corrosive than other acids and is inexpensive compared with other mineral acids. Additionally, phosphoric salts generated by neutralization at the end of the pretreatment process can be utilized as fertilizers, fermentation buffers, and for microorganism nutrition (Premjet et al., 2016; Satari et al., 2019). This study aimed to determine the optimal dose of H_3PO_4 for

pretreating weed biomass (*V. pusilla*) to maximize glucose recovery and bioethanol production.

Materials and methods

Material

The above-ground biomass of *V. pusilla* was collected from a naturally occurring location in the Wang Thong district of Phitsanulok, Thailand (16°51′03.3″N, 100°44′38.2″E). The herbarium in Naresuan University's biology department confirmed the authenticity of the material. The specimen was preserved with record code 05868 for reference purposes at the herbarium.

Sample preparation

The *V. pusilla* sample was rinsed with tap water to remove soil debris and dried under shade for five days. Subsequently, they were cut into long pieces (approximately 5 cm) and crushed into powder using a milling machine (Retsch, Haan, Germany). They were then sieved through a 150–300 µm laboratory test sieve before being placed in bottles at 25 ℃ for future analysis and experiments. (Hames et al., 2008).

Chemical composition analysis

We followed the technique of compositional analysis outlined by Premjet et al., 2018a. The chemical compositions of untreated and treated samples, including monomer sugar, lignin, ash, and extractives, were examined using the National Renewable Energy Laboratory (NREL) development method (Premjet et al., 2018a; Sluiter et al., 2008a; Sluiter et al., 2012; Sluiter et al., 2008b).

Analytical Processes

The details of these analytical methodologies are described in a previous report by Premjet et al., 2018a. Ultraviolet–visible (UV-Vis) spectrophotometer (Analytik Jena Specord 40, Analytik Jena AG, Jena, Germany) absorbance at 205 nm was utilized to assess acid-soluble lignin.

Monomeric sugar, and ethanol were analyzed using a high-performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies, Waldbronn, Germany) system integrated with a refractive index detector (G1362A; Agilent Technologies, Waldbronn, Germany) and a Bio-Rad Aminex-HPX 87H column (300 mm × 7.8 mm; Hercules, CA, USA), which were maintained at 55 and 60 °C, respectively. The mobile

phase consisted of 0.005 M sulfuric acid with a 0.6 mL/min flow rate. The injection volume was 20 µL (Premjet et al., 2022).

Phosphoric Acid Pretreatment

V. pusilla lignocellulosic feedstock was pretreated according to the procedure specified by Premjet et al., 2022. The raw material (3 g) was mixed with different concentrations of H_3PO_4 solution (70%, 75%, 80%, and 85% v/v) in a polypropylene tube at a solid-to liquid ratio of 1:8. The tube carrying the mixture was then sealed and heated for 60 min at 60 °C in a water bath. The reaction was terminated by adding 25 mL acetone and stirring with a glass rod. The liquid mixture was separated using a fixed-angle centrifuge at 7100× g for 15 min at 15 °C. The supernatant was then discarded. This procedure was carried out at least twice, and the solid fraction was rinsed with distilled water until the pH was close to 7.0

Equations (1) and (2) were used to evaluate the recovery yield (%) and lignin removal (%) (Obeng et al., 2019):

$$\text{Recovery yield (\%)} = \frac{\text{Solid recovery of each content (\%)} \times \text{Treated composition of each content (\%)}}{\text{Untreated composition of each content (\%)}} \quad (1)$$

$$\text{Lignin removal (\%)} = 100\ \% - \text{lignin recovery} \quad (2)$$

Enzymatic Hydrolysis

The saccharification hydrolysis of both the pretreated and raw samples was evaluate using a previously described method (Obeng et al., 2019). A 50 mL Erlenmeyer flask containing 10 mL of digestion solution was used to hydrolyze 100 mg of biomass (dry weight) from both the raw and pretreated samples. The enzyme loadings for 30 filter paper units (FPU) of cellulase (*Trichoderma reesei* C2730, Sigma-Aldrich, St. Louis, MO, USA) and 60 U-glucosidase (Oriental Yeast Co., Ltd., Tokyo, Japan) per gram of dry biomass in a sodium citrate buffer (pH 4.8) with 2% (w/v) sodium azide. The flask of each reaction was incubated at 50 °C and 150 rpm for 96 h in a rotary shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA).

The hydrolysate solution (200 µL) was collected after 12, 24, 48, 72, and 96 h for fermentable sugar analysis (Obeng et al., 2019).

The hydrolysis efficiencies of glucose (HEG) and glucose recovery (GR) were determined using the following equations from Obeng et al. (Obeng et al., 2019):

$$\text{HEG (\%)} = \frac{\text{Glucose generated by hydrolysis (g)} \times 100}{1.11 * \text{Initial glucan in substrate (g)}} \quad (3)$$

$$\text{GR (\%)} = (\text{solid recovery} \times \text{glucan in substrate} \times 1.11 \times \text{HEG}) \times 100 \quad (4)$$

Where 1.11 is the conversion factors for glucan to glucose.

Determination of Biomass Microstructure

The analytical techniques had been followed in a previous investigation (Obeng et al., 2019; Obeng et al., 2021). The raw and treated *V. pusilla* feedstocks were freeze-dried and fixed on aluminum stumps using double-sided carbon tape and a gold coating. A field emission scanning electron microscope (FESEM; Thermo Fisher Scientific, Apero S, Waltham, MA, USA) was used to observe modifications in the surface morphology of all specimens (Obeng et al., 2019; Obeng et al., 2021).

Crystallinity of Biomass

Changes in the crystallinity index (CrI) of raw and treated samples were determined by X-ray diffraction (XRD) using a PANalytical X'pert Pro, PW 3040/60 diffractometer (Almelo, The Netherlands). The biomass samples underwent a triple wash with acetone and were thereafter subjected to drying at a temperature of 32 °C. The dry specimens were pulverized to achieve a particle size that passed through a 150 µm mesh screen. The diffraction intensity was determined within the angular range of 2θ = 10–40°, using a scanning rate of 0.02° s^{-1}/min (Obeng et al., 2019; Obeng et al., 2021).

Using the Segal equation, the CrI was determined as follows (Segal et al., 1959):

$$CrI = (I_{002} - I_{am}) / I_{002} \times 100\% \quad (5)$$

Where I_{am} is the lowest intensity of the amorphous sector at 2θ = 18.0° and I_{002} is the greatest intensity of the crystallinity sector at 2θ = 22.0°.

Microorganisms

In this study, bioethanol was produced using *Saccharomyces cerevisiae* TISTR 5339. These yeast strains were purchased in special order and submitted to the Thailand Institute of Science and Technology (TISTR).

Preparation of Seed Culture

A single loop from yeast strain, *S. cerevisiae* TISTR 5339 was used as the initial inoculum. *S. cerevisiae* TISTR 5339 was placed into a 50 mL Falcon tube with 10 mL of yeast malt medium and incubated with a rotating shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA) at 30 °C for 18 h.

Preparing Biomass Hydrolysate for Ethanol Production

The biomass hydrolysate (BH) was prepared according to procedures outlined in a previous study (Premjet et al., 2018b). After the *V. pusilla* biomass was treated under optimal conditions, followed by enzymatic saccharification, the enzymatic activity was terminated by heating the BH to 100 °C in a water bath (Grant Instrument, Grant W28, Ltd. Barrington, Cambridge, UK) for 20 min. The solid components of the biomass were separated by centrifugation at 12,000× *g* for 2 h before being filtered through a glass microfiber filter (Whatman, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK). The BH was then concentrated using a rotary vacuum evaporator (Heidolph Instruments, Hei-VAP Advantage, GmbH & Co. KG Walpersdorfer Str., Schwabach, Germany) until it yielded approximately 20 g/L glucose. The pH of the BH was adjusted to 6 using a 1 M NaOH solution and chilling overnight at 4 °C. Subsequently, the mixture was centrifuged for 2 h at 12,000× *g* and passed through a glass filter. The BH was subsequently maintained at 4 °C for use in future experiments (Premjet et al., 2018b).

Medium for Bioethanol Production

Ethanol was produced using the control and the BH media. The BH medium contained glucose derived from the biomass, whereas commercial glucose was used as the carbon source in the control medium. Each medium was prepared by adding 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 2 g/L $MgSO_4$, and 2 g/L K_2HPO_4, and the pH was set at 6 (Premjet et al., 2018b).

In this study, all liquid culture media were prepared under aseptic conditions and passed through a Millipore filter with a 0.2 μm pore size (Merck Ltd. Tullagreen, Carrigtwohill, Co., Cork, Ireland).

Ethanol Production

The specifications of the bioethanol production methodology were followed in a previous study by Premjet et al., 2018b. The production of bioethanol utilized 50 mL of both BH and a control medium. Both media were inoculated with 2% (v/v) *S. cerevisiae* TISTR 5339 seed culture and incubated at 30 °C on a rotary shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA) at 150 rpm for 24 h. A milliliter of the liquid fraction was collected for HPLC analysis after 3, 6, 9, 12, 15, 18, 21, and 24 h of incubation to assess glucose consumption and ethanol production (Premjet et al., 2018b). The ethanol yield was determined using the following equation:

$$\text{Ethanol yield (\%)} = \frac{\text{Ethanol in fermentation liquid (g)} \times 100}{0.511 \times \text{Glucose initial (g)}} \quad (6)$$

Determination Growth of Microorganisms

The optical density at 600 nm was determined using a UV spectrophotometer to monitor the yeast cell growth (Premjet et al., 2018b). At 600 nm, an OD of 1.0 corresponds to approximately 1.5×10^7 cells/mL.

Statistical Analysis

Each experiment was performed in triplicate; statistical evaluation of data was performed by analysis of variance using the SPSS version 26.0. The statistically significant difference of each measurement was assessed utilizing Duncan's test ($p < 0.05$). The result is expressed as the average and standard deviation (± SD)

Results

Compositional Analysis

The lignocellulosic *V. pusilla* biomass was used as an alternative biomass source. The chemical composition of the above-ground *V. pusilla* biomass consisted of three primary elements: cellulose, hemicellulose, and lignin (Table 4). The major component of carbohydrates is cellulose (48.1 ± 0.3%), mainly in the form of glucan, followed by hemicellulose composed of xylan (19.2 ± 0.4%) and arabinan (1.2 ± 0.1%). The total amount of carbohydrates was approximately 70%. The lignin content was composed of acid-insoluble lignin (AIL, 23.5 ± 0.1%) and acid-soluble lignin (ASL, 4.4 ± 0.1%). However, the ash and extractive values were 6.1 ± 0.2% and 18.2 ± 0.2%, respectively.

Table 4 Chemical composition of *V. pusilla*

Composition (% dw)	*V. pusilla* (%)
Glucan	48.1 ± 0.3
Xylan	19.2 ± 0.4
Arabinan	1.2 ± 0.1
Ash	6.1 ± 0.1
Extractive	18.2 ± 0.2
AIL*	23.5 ± 0.1
ASL**	4.4 ± 0.1
Total lignin	27.9 ± 0.2

Note: AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin

Impact of H_3PO_4 on the Chemical Composition of *V. pusilla* Biomass

To obtain monomer sugar from weed biomass, the *V. pusilla* biomass was treated with 70%, 75%, 80%, and 85% H_3PO_4. The major components of this biomass, before and after pretreatment, are listed in Table 5. After pretreatment, the concentration of H_3PO_4 had a substantial impact on changes in glucan, xylan, and lignin contents. The xylan content of this biomass decreased by 7.6 ± 0.4%, 4.8 ± 0.3%, and 4.0 ± 0.2% after pretreatment with 70%, 75%, and 80% H_3PO_4, respectively. However, the arabinan and xylan content was eliminated by treatment with 70–85% and 85% H_3PO_4, respectively (Table 5).

Increasing the H_3PO_4 concentration led to a decrease in the AIL, ASL, and total lignin contents (Table 5). Additionally, the total lignin content was reduced from 27.9 ± 0.2% (raw material) to 10.5 ± 0.7%. This accounted for approximately 80% of the total lignin removal. However, the degree of delignification gradually improved to 54.4 ± 1.1%, 68.0 ± 1.2%, 71.2 ± 0.5%, and 82.4 ± 1.2% when treated with 70%, 75%, 80%, and 85% H_3PO_4, respectively (Table 5).

Further, the glucan recovery declined steadily to 79.9 ± 0.4%, 78.3 ± 1.2%, 74.7 ± 0.7%, and 68.9 ± 0.7% after pretreatment with 70%, 75%, 80%, and 85% H_3PO_4, respectively (Table 5). In contrast, the relative glucan content improved significantly ($p < 0.05$) to 71.4 ± 0.3% and 73.6 ± 1.1% after pretreatment with 70% and 75% H_3PO_4, respectively (Table 5). Subsequently, with increase in the concentration of H_3PO_4 to 80% and 85%, the relative glucan content decreased to 71.5 ± 0.7% and 70.8 ± 0.8%, respectively; however, it was not significantly different.

The solid recovery yield gradually decreased with an increase in the concentration of H_3PO_4. However, pretreatment with 75% and 80% H_3PO_4 did not significantly ($p < 0.05$) affect the solid recovery yield, which was 51.1 ± 0.7% and 50.2 ± 0.4%, respectively (Table 5). The lowest solid recovery yield (46.8 ± 1.7%) was achieved by pretreating with 85% H_3PO_4.

Table 5 Composition of *V. pusilla* after H_3PO_4 pretreatment

Composition (% dw)	Raw material	Concentration of H_3PO_4 (%)			
		70	75	80	85
Glucan	48.1±0.3^{c}	71.4±0.3^{b}	73.6±1.1^{a}	71.5±0.7^{b}	70.8±0.8^{b}
Xylan	19.2±0.4^{a}	7.6±0.4^{b}	4.8±0.3^{c}	4.0±0.2^{d}	n.d.
Arabinan	1.2±0.1^{a}	n.d.	n.d.	n.d.	n.d.
AIL*	23.5±0.1^{a}	21.0±0.6^{b}	14.8±0.7^{c}	13.4±0.3^{d}	8.1±0.7^{e}
ASL**	4.4±0.1^{a}	2.7±0.1^{b}	2.7±0.0^{b}	2.6±0.0^{b}	2.5±0.0^{c}
Total lignin	27.9±0.2^{a}	23.7±0.6^{b}	17.5±0.7^{c}	16.0±0.3^{d}	10.5±0.7^{e}
Solid recovery	100±0.0^{a}	53.8±1.4^{b}	51.1±0.7^{c}	50.2±0.4^{c}	46.8±1.7^{d}
Glucan recovery	100±0.0^{a}	79.9±0.4^{b}	78.3±1.2^{c}	74.7±0.7^{d}	68.9±0.7^{e}
Xylan recovery	100±0.0^{a}	21.4±1.2^{b}	12.8±0.9^{c}	10.4±0.6^{d}	n.d.
Arabinan recovery	100±0.0^{a}	n.d.	n.d.	n.d.	n.d.
AIL recovery	100±0.0^{a}	48.0±1.4^{b}	33.1±0.2^{c}	28.6±0.6^{d}	16.0±1.5^{e}
ASL recovery	100±0.0^{a}	32.7±0.8^{b}	30.6±0.2^{c}	29.9±0.1^{d}	26.0±0.3^{e}
Total lignin recovery	100±0.0^{a}	45.6±1.1^{d}	32.0±1.2^{c}	28.8±0.5^{d}	17.6±1.2^{e}
Total lignin removal	n.d.	54.4±1.1^{d}	68.0±1.2^{c}	71.2±0.5^{b}	82.4±1.2^{a}

Note: AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin

The superscripted characters within rows indicate the statistical significance of the differences between them ($p < 0.05$), n.d. = not determined

Enzymatic Saccharification of *V. pusilla* Biomass

To evaluate the influence of H_3PO_4 concentration on GR and HEG, cellulose generated after pretreatment with 70%, 75%, 80%, and 85% H_3PO_4. was utilized as a substrate for enzymatic hydrolysis, as shown in Figures 4A,B. After treating raw materials with different concentrations of H_3PO_4., the yields of GR and HEG increased significantly ($p < 0.05$). During enzymatic saccharification, the GR and HEG yields improved considerably after 12 h, and then steadily over the next 24, 48, 72, and 96 h. The highest GR and HEG yields were observed in both untreated and treated samples after 96 h of incubation. At H_3PO_4 concentrations of 70%, 75%, 80%, and 85%, GR yields were 31.0 ± 0.2%, 40.8 ± 0.3%, 38.9 ± 0.1%, and 36.4 ± 0.1% (Figure 4A),

whereas HEG yields were 65.4 ± 0.3%, 88.0 ± 0.6%, 88.0 ± 0.1%, and 89.0 ± 0.2%, respectively (Figure 4B). The HEG yields with 75% H_3PO_4 (88.0 ± 0.6%) and 80% H_3PO_4 (88.0 ± 0.1%) did not differ significantly, whereas the greatest HEG yield (89.0 ± 0.2%) was observed with 85% H_3PO_4. Furthermore, this feedstock was pretreated with 70% and 75% H_3PO_4, resulting in a substantial increase in GR yields (31.0 ± 0.2% and 40.8 ± 0.3%, respectively); however, GR yields declined marginally when H_3PO_4 concentration was increased to 80% and 85%. Moreover, the lowest yield of HEG (19.4 ± 0.0%) and GR (11.5 ± 0.0%) were obtained from untreated sample (Figure 4A,B). The yields of GR and HEG were approximately 3.5 and 4.5 times greater, respectively, than those of the raw material. This demonstrates that 75% H_3PO_4 is the optimal pretreatment for *V. pusilla* feedstock.

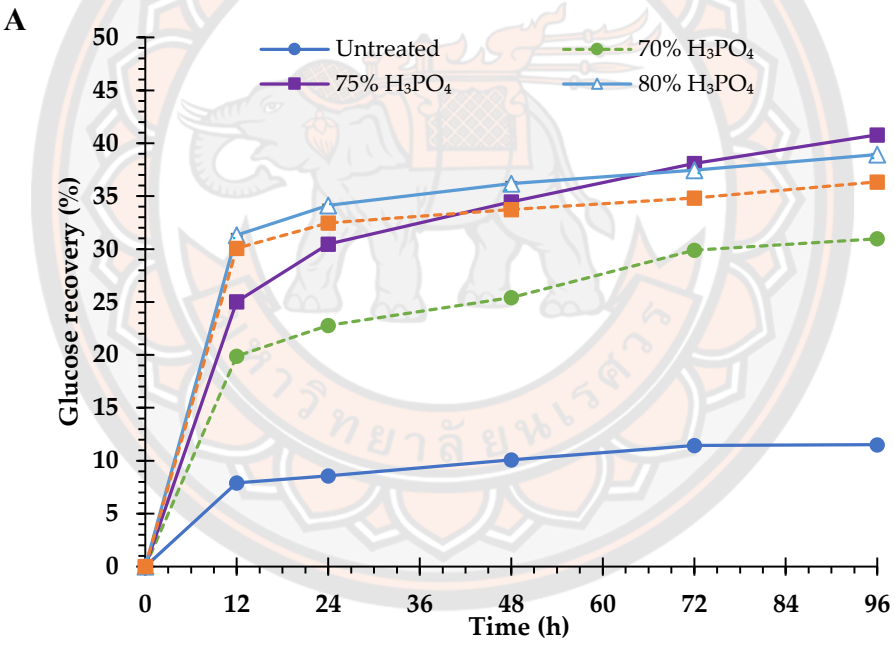

Figure 4 (A) Glucose recovery and (B) hydrolysis efficiency of untreated and treated biomass of *V. pusilla*

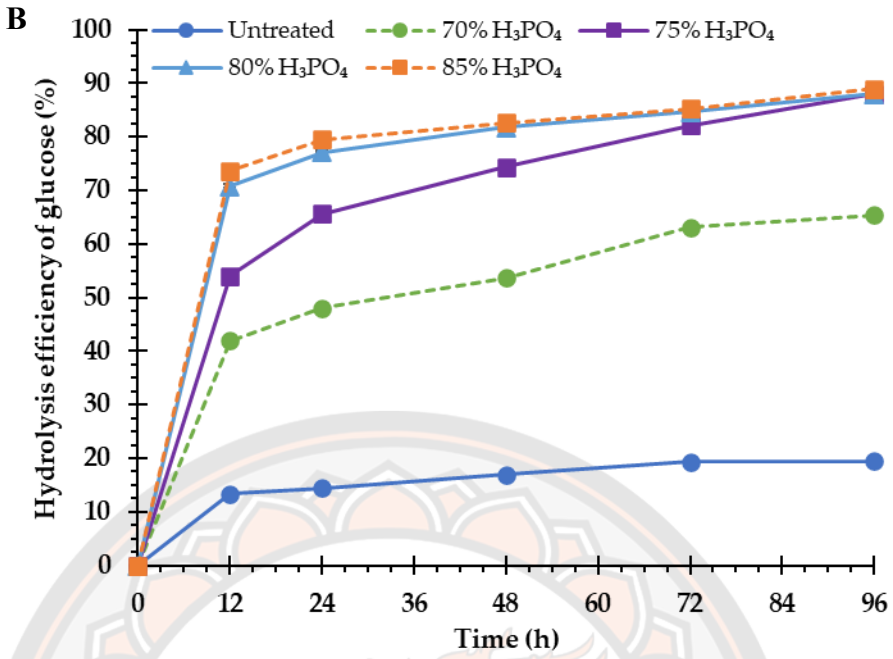

Figure 4 (cont.)

Scanning electron microscopy assessment

To gain a more comprehensive understanding of the relationship between biomass morphology and the ability of cellulase to degrade cellulose, the surface morphology of untreated and treated samples (with varied concentrations of H_3PO_4) of *V. pusilla* was evaluated using scanning electron microscopy (SEM), as shown in Figure 5A–E. The SEM images of untreated samples revealed a highly compressed fibril structure arranged in strict bundles on the surface of the *V. pusilla* biomass (Figure 5A). After pretreatment with 70% and 75% H_3PO_4, the surface structure displayed more fractures, peeling, and fiber deconstruction. Consequently, the fibers gradually split until severely disordered, resulting in increased porosity (Figure 5B–C). Surface deconstruction increased with an increase in H_3PO_4 concentration. When treated with 80% H_3PO_4, the fibrils disintegrated and sustained additional damage (Figure 5D), and pretreatment with 85% H_3PO_4 led to collapse of the structure (Figure 5E).

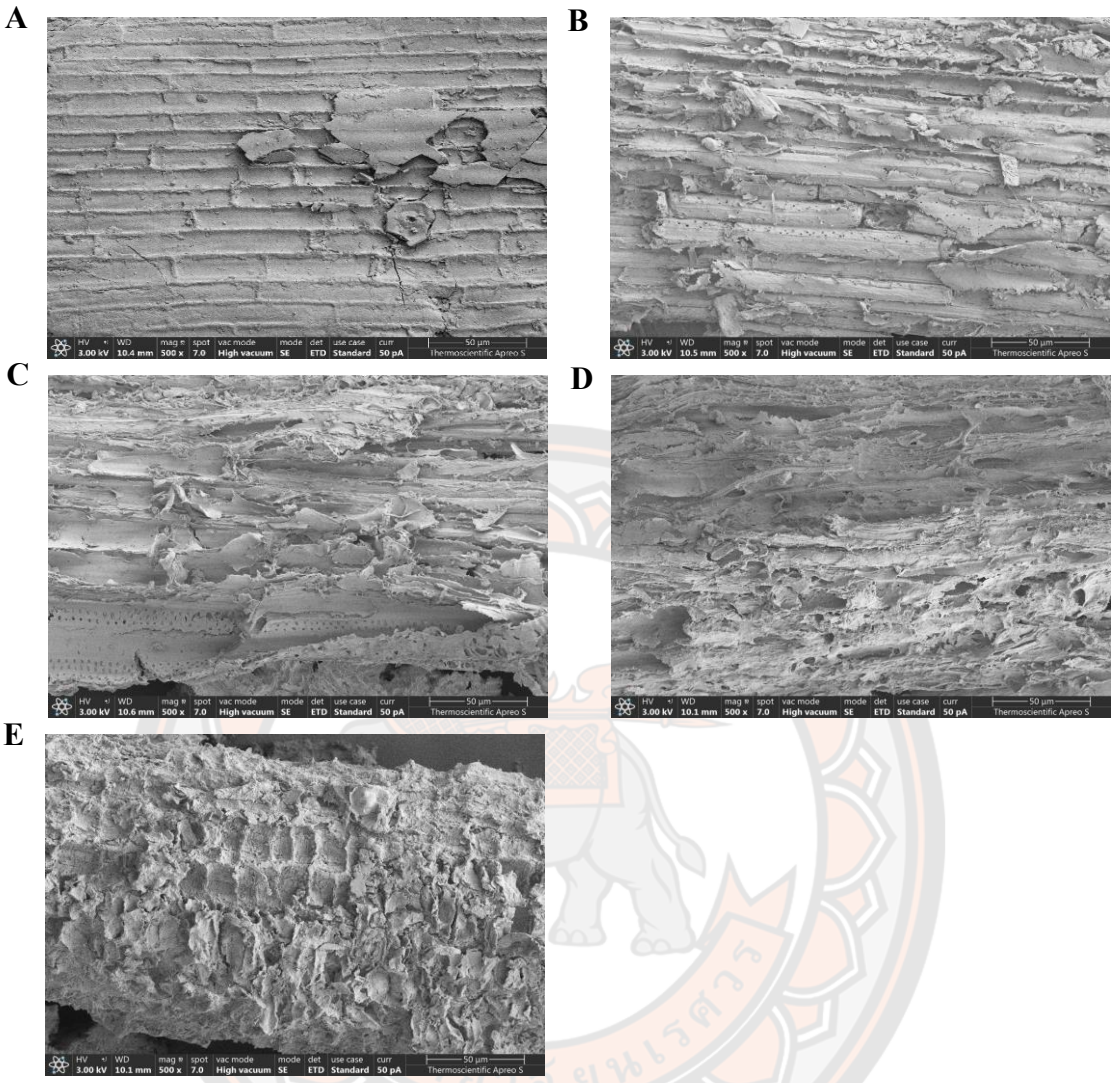

Figure 5 Surface morphology of (A) untreated and pretreated *V. pusilla* samples with (B) 70%, (C) 75%, (D) 80%, and (E) 85% H_3PO_4

Effects of H_3PO_4 on Cellulose Crystalline Structure

H_3PO_4 treatment on the crystalline cellulose of *V. pusilla* feedstock restricted cellulose digestibility. Therefore, the CrI of the untreated and treated samples was determined by operating an X-ray diffractometer (XRD). Cellulose crystallinity was altered after treatment with varying H_3PO_4 concentrations. The X-ray diffractogram pattern of the untreated sample was identified as cellulose I crystallinity that featured two major peaks at $2\theta = 15.5°$ and $2\theta = 22.0°$ (Figure 6), with a CrI value of 59.5% (Table 6). After the sample was treated with 70% H_3PO_4, the highest CrI value (65.0%) was observed. The CrI values were lowered to 63.5%, 49.1%, and 42.9% when the material was treated with 75%, 80%, and 85% H_3PO_4, respectively. With 70% and 75% H_3PO_4 (Table 6), the XRD patterns were still indicative of cellulose I crystallinity. However, when the sample was treated with 80% H_3PO_4, the initial transition from cellulose I to cellulose II was identified because the heights of the two significant peaks of crystalline cellulose diminished more. However, XRD patterns revealed that cellulose I was fully converted into cellulose II crystallinity after pretreatment with 85% H_3PO_4 (Figure 6).

Table 6 Crystallinity index of untreated and H_3PO_4 pretreated samples

	Raw material	Concentration of H_3PO_4 (%)			
		70	**75**	**80**	**85**
CrI (%)	59.5	65.0	63.5	49.1	42.9

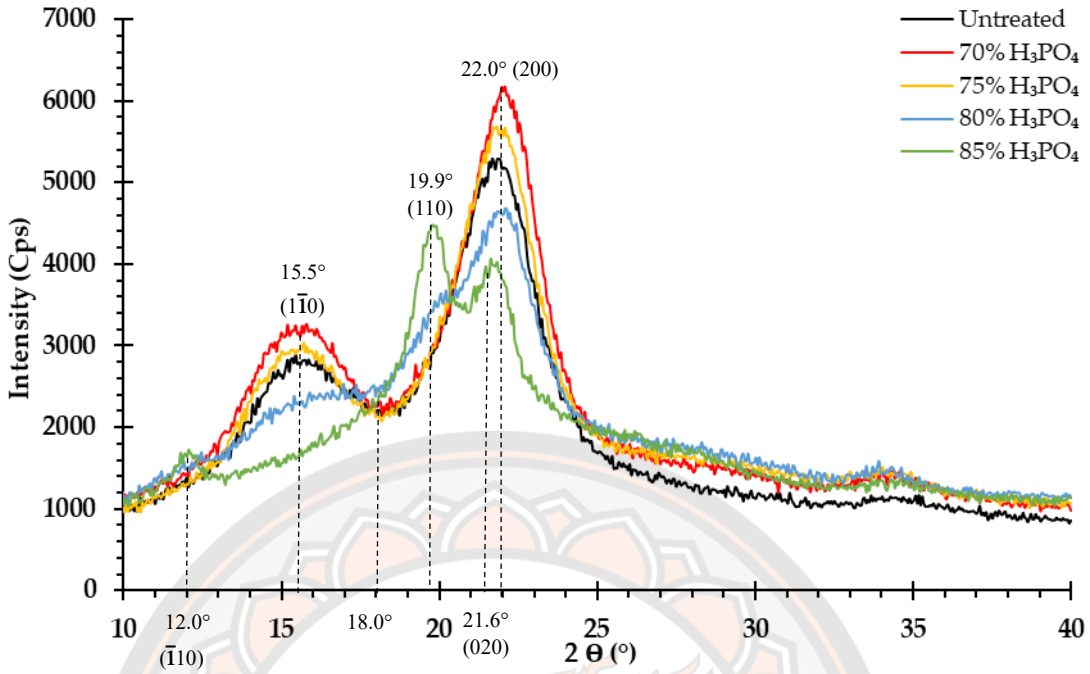

Figure 6 X-ray diffraction pattern of untreated and H_3PO_4 pretreated samples of *V. pusilla*

Ethanol Fermentation

The BH medium was produced by enzymatic hydrolysis of *V. pusilla* biomass after pretreatment with 75% H_3PO_4. In this experiment, a non-detoxified BH medium containing 20.0 g/L glucose was employed as the culture medium for cellulosic ethanol by *S. cerevisiae* TISTR 5339. The control group consisted of a standard (ST) medium with 20.0 g/L D-glucose (reagent grade, 98%). The patterns of bioethanol formation, yeast cell growth, and glucose consumption in both media are shown in Figures 7 and 8. According to the results, glucose consumption in both ST and BH media gradually decreased and was completely consumed after 15 h of incubation. Ethanol production was observed in the ST and BH media after 6 and 9 h of incubation, respectively. Subsequently, the ethanol yields in both media steadily increased. The maximum ethanol yields of 9.4 g/L (92.1%) and 8.9 g/L (87.5%) were obtained from the ST and BH media after 15 h of incubation, respectively. After this stage, ethanol production by the yeast in both media did not increase. However, ethanol production in the BH medium was lower than that in the ST medium (Figure 7). Furthermore, the yeast growth rate profiles in both media were comparable. The maximum growth rate was

observed in both media after 12 h of incubation. However, the growth rate of the yeast in the ST medium was greater than that in the BH medium (Figure 8).

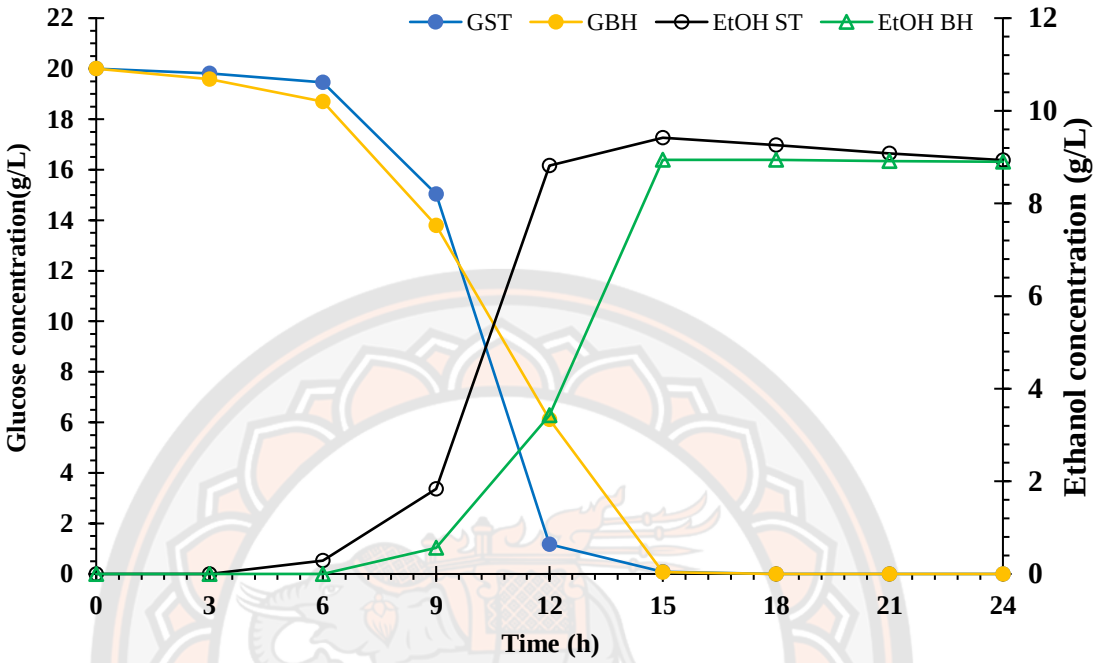

Figure 7 Profiles of glucose consumption and bioethanol fermentation of S. *cerevisiae* TISTR 5339

Note: GST = Glucose consumption in standard medium, GBH = glucose consumption in biomass hydrolysate medium, EtOH ST = ethanol in standard medium, and EtOH BH = ethanol in biomass hydrolysate medium

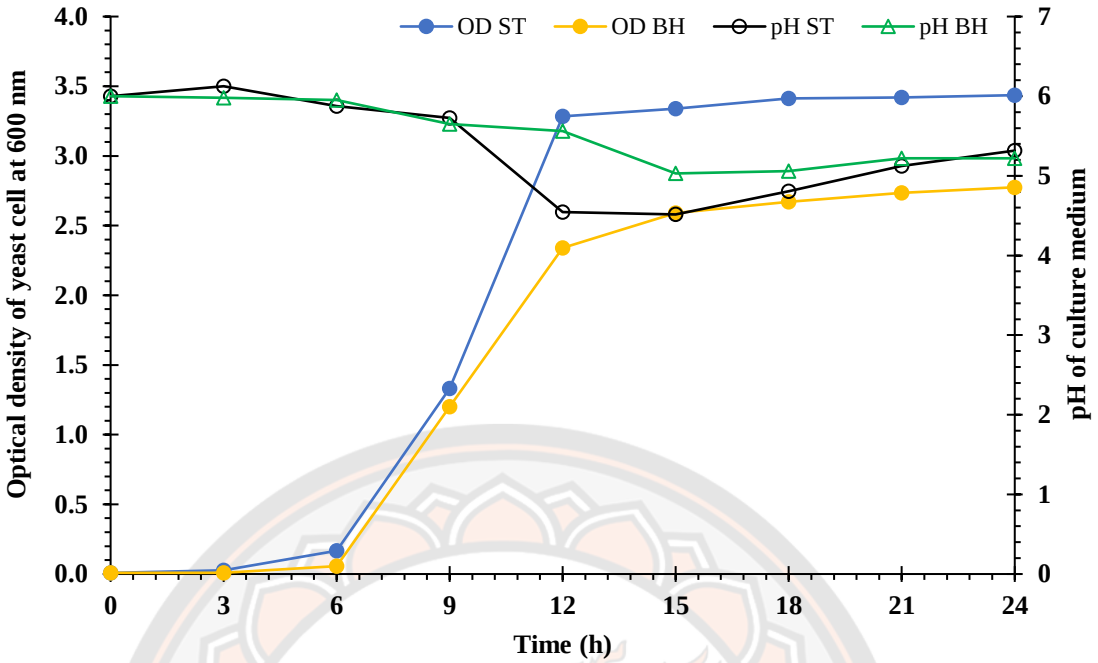

Figure 8 Cell growth of *S. cerevisiae* TISTR 5339

Note: OD ST = optical density of yeast cell in standard medium, OD BH = optical density of yeast cell in biomass hydrolysate medium, pH ST = the pH in standard medium, and pH BH = the pH in biomass hydrolysate medium

Discussion

Compositional Analysis

According to our results, *V. pusilla* biomass has a higher glucan content than that of various lignocellulosic materials promoted for bioethanol manufacturing, such as spruce (47.1%), pine (45.6%) (Yu et al., 2017), barley husk (45.7%) (Fortunati et al., 2016), Douglas fir (46.1%) (Zhu & Yadama, 2018), corn cob (45.0%) (Louis & Venkatachalam, 2020), bamboo (44.6%) (Li et al., 2015), sugarcane bagasse (43.7%) (Vanitjinda et al., 2019), wheat straw (42.5%) (Tsegaye et al., 2019a), cotton stalk (42.3%) (Wang et al., 2019), rice straw (41.9%) (Zhang et al., 2020a), and eucalyptus (41.4%) (Wang et al., 2018). In addition, the glucan content was higher than that of weed lignocellulosic biomass, including *Sida acuta* (46.9%), *Achyranthes aspera* (45.9%) (Premjet et al., 2016), *Prosopis juliflora* (45.5%), *Lantana camara* (45.1%), *Saccharum spontaneum* (45.1%), and Siam weed (41.0%) (Kumar et al., 2020). Regarding the amount of xylan content, it was greater than that observed in sponge gourd fibers (17.4%), banana waste (14.8%) (Kumar & Sharma, 2017), cotton (16.0%), acacia (13.0%) (Kim et al., 2016a), and Japanese cedar (13.8%) (Hassan et al., 2018). In addition, the lignin content of *V. pusilla* was equivalent to that of rubber wood (27.6%) (Khan et al., 2018) but lower than that of several lignocellulosic biomasses promoted for biofuel production, such as oak (35.4%), Japanese cedar (33.5%) (Hassan et al., 2018), switchgrass (31.2%) (Bonfiglio et al., 2021), poplar (29.1%), and hemlock (28.5%) (Hassan et al., 2018). These results indicate that the lignocellulosic *V. pusilla* biomass has high potential for application in sugar platform-based biorefineries for the manufacture of cellulosic ethanol as well as other importance compounds.

Impact of H_3PO_4 on the Chemical Composition of *V. pusilla* Biomass

The exploitation of lignocellulosic biomass in sugar-based biorefineries requires a pretreatment procedure to break down its recalcitrant structure (Bhatia et al., 2020; Mahmood et al., 2019; Premjet et al., 2022). It has been observed that acid pretreatment with H_3PO_4 is an extremely successful method for breaking glycosidic links in lignocellulose and dissolving hemicellulose, cellulose, and a small quantity of lignin (Sathitsuksanoh et al., 2012; Woiciechowski et al., 2020; Zhao et al., 2022). The results indicated that H_3PO_4 affected both partial and entire reduction of xylan from the

biomass. Under acidic conditions, hemicellulose is more sensitive to solubility than lignin and cellulose because it is amorphous, has a low degree of polymerization, and is predominantly present in the form of xylan (Chen et al., 2022; Liu et al., 2018; Satari et al., 2019; Zoghlami & Paës, 2019). In contrast, lignin is an amorphous, water-insoluble heteropolymer composed of three cinnamyl alcohol precursors (sinapyl alcohol, coniferyl alcohol, and p-coumaryl alcohol) connected via various links and packed into a lignin layer in the plant cell wall, resulting in resistance to chemical pretreatment (Arora et al., 2020; Kucharska et al., 2018; Yoo et al., 2020). Consequently, even pretreatment of this biomass with the maximum concentration of H_3PO_4 (85%) resulted in only partial removal of lignin. Nevertheless, both partial xylan and lignin removal led to an increase in relative glucan content in solid recovery after pretreatment of this biomass with 70%–80% H_3PO_4 (Obeng et al., 2018; Premjet et al., 2022). However, pretreatment of feedstock with 85% H_3PO_4 led to breakdown of the linkages of the lignin-carbohydrate complex and dissolution of cellulose fibrils and hemicellulose due to destruction of hydrogen bonds between sugar chains, leading to a substantial reduction in solid recovery, glucan recovery, relative glucan content, and elimination of xylan content (Satari et al., 2019; Sathitsuksanoh et al., 2013). Furthermore, similar effects of H_3PO_4 have been observed in various feedstocks, including *Luffa cylindrica* (Wang et al., 2016), *A. aspera* and *S. acuta* (Premjet et al., 2016), *Hibiscus sabdariffa* (Premjet et al., 2018a), *Durio zibethinus* (Obeng et al., 2018), and *Hibiscus cannabinus* (Premjet et al., 2022).

Enzymatic Saccharification of *V. pusilla* Biomass

The primary goal of lignocellulosic biomass pretreatment is the elimination of hemicellulose and lignin, leaving cellulose in the solid phase for subsequent enzymatic hydrolysis and fermentation (Zhao et al., 2022). When raw *V. pusilla* biomass was pretreated with varying concentrations of H_3PO_4, the GR and HEG yields were considerably enhanced for each pretreated sample depending on the concentration of H_3PO_4. These results demonstrate that the effect of H_3PO_4 pretreatment on the hydrolysis of each pretreated sample in relation to glucose was more pronounced. In contrast, enzyme hydrolysis of the raw material resulted in the lowest yields for GR and HEG. This is because of the stiffness and obstinacy of lignocellulosic feedstock, which

inhibits enzyme hydrolysis (Bhatia et al., 2020; Chen et al., 2022; Kumar et al., 2020; Zoghlami & Paës, 2019).

During the conversion processes, lignin in lignocellulosic biomass functions as an inhibitor by preventing enzyme accessibility to cellulose and inhibiting enzymatic activity through nonproductive binding. Consequently, eliminating lignin from lignocellulosic feedstock via acidic pretreatment is crucial to minimize chemical and physical barriers and enhance the access of cellulolytic enzymes to cellulose (Satari et al., 2019; Yoo et al., 2020). These results demonstrate that increasing the concentration of H_3PO_4 positively affects both GR and HEG yields, which coincides with the elimination of AIL and ASL. The greatest GR and HEG yields were obtained when the feedstock was pretreated with 75% H_3PO_4, whereas AIL (66.9 ± 0.2%) and ASL (69.4 ± 0.2%) were excluded. These quantities represent the total amount of lignin (68.0 ± 1.2%) extracted from the treated biomass. These results reveal that the increase in the yields of GR and HEG resulted from partial delignification due to H_3PO_4 pretreatment. In addition, delignification often causes the breakdown of the polymeric network, leading to increased porosity and diminished enzyme inhibitory action (Pihlajaniemi et al., 2016; Premjet et al., 2022; Satari et al., 2019). According to reports, pretreatment of various lignocellulosic biomasses using particularly aggressive methods, such as harsh bases or acids, accelerates delignification of the feedstock and disintegration of carbohydrates (Yuan et al., 2021). This suggests that complete lignin elimination is not required.

The hemicelluloses in lignocellulosic biomass are physical barriers surrounding cellulose, limiting hydrolysis reaction, restricting enzyme access to cellulose, and lowering cellulase activity. However, lignin had a greater effect than hemicellulose. Therefore, removing hemicellulose present in the xylan content may accelerate cellulose hydrolysis by expanding the exposure of cellulase to cellulose (Bhatia et al., 2020; Chen et al., 2022; Kumar et al., 2020; Zoghlami & Paës, 2019), which is comparable to lignin. In this operation, hemicellulose was effectively eliminated from this feedstock, which was treated with 85% H_3PO_4. The glucose HEG yields of samples pretreated with 75% and 80% H_3PO_4 were not significantly different. The samples treated with 75% H_3PO_4 resulted in the highest GR (40.8 ± 0.3%), whereas those treated with 85% H_3PO_4 resulted in the highest HEG rates (89.0 ± 0.2%). Although the HEG

is maximum under certain circumstances, such as at a concentration of 85% H_3PO_4 in this study, the total GR may be lower due to high solid loss (53.2 ± 1.7% in this study) under severe conditions (Premjet et al., 2018b; Yoo et al., 2017). Consequently, the entire GR process defines the pretreatment parameters used. This study confirmed that pretreatment of *V. pusilla* biomass with 75% H_3PO_4 was optimal condition. At this concentration, approximately 78.3 ± 1.2% and 51.1 ± 0.7% of glucan and solid, respectively, were recovered. This demonstrates that more than 50% of glucan in the treated biomass was still present. According to reports, the ideal pretreatment condition is one in which the GR level is the greatest and closest to the initial glucan content of the biomass (Obeng et al., 2018; Yoo et al., 2017). The increase in both GR and HEG yields may be attributed to the disintegration and dissolution of hemicellulose and lignin, which renders cellulose present in the treated biomass of *V. pusilla* vulnerable to cellulases. In comparison to other lignocellulosic biomasses, the HEG yield of the feedstock was higher than that of vegetable hummingbird (86.0%), alamo switchgrass (85.0%), eastern gamagrass (80.5%), switchgrass (73.5%) as reported by Satari et al. (Satari et al., 2019), Devil's horsewhip (86.2%), common wire-weed (82.2%) (Premjet et al., 2016), pine (80.0%), and Douglas fir (79.0%) (Hossain et al., 2020). The GR was also greater than that of Chanee durian peel (38.5%) (Obeng et al., 2018), Douglas fir (31.2%), and pine (28.0%) (Hossain et al., 2020).

SEM assessment

To accelerate the enzymatic conversion of lignocellulose to monomeric sugar, the innate stiffness of plant cell walls must be disassembled by processing using acids or bases to improve the access of enzymes to the cellulose surface (Kumar et al., 2020; Mahmood et al., 2019; Yoo et al., 2020). The raw material exhibited the structural obstinacy of *V. pusilla*, which is predominantly composed of a lignin-carbohydrate matrix, rendering it extremely resistant to enzymatic hydrolysis (Bhatia et al., 2020; Zoghlami & Paës, 2019). The structural transformation of the treated sample was accompanied by a substantial alteration in its chemical composition upon treatment with 70% and 75% H_3PO_4. This is due to H_3PO_4 pretreatment which enabled the breakdown of the resistant lignocellulose structure by destroying hemicellulose and lignin networks and increasing cellulose accessibility. Consequently, modifications in

the internal cell wall composition, such as destruction, increased porosity, and loss of recalcitrant structure, increase enzyme accessibility to cellulose (Ji et al., 2015; Satari et al., 2019). These outcomes are consistent with the steady increase in the GR, HEG yields and contribute to the maximum GR, and HEG generated by 75% H_3PO_4 pretreatment. Furthermore, the SEM micrographs of both treated samples revealed greater extent of degradation of the morphological surface when H_3PO_4 concentration was increased to 80% and 85%. This effect is caused by an upsurge in cellulose degradation under harsh conditions, resulting in a considerable decrease in GR yields (Chu et al., 2018; Gourlay et al., 2012; Ji et al., 2015; Luo et al., 2019; Moxley et al., 2008; Mund et al., 2017; Satari et al., 2019). In a previous study, we found that eliminating lignin and hemicellulose, which are physical barriers surrounding cellulose, could be a key factor contributing to the improvement in HEG and GR yields (Premjet et al., 2022). These findings validate outcome of the enzymatic hydrolysis.

Effects of H_3PO_4 on Cellulose Crystalline Structure

Cellulose is a linear polysaccharide composed of highly organized repeating glucose units. These glucan chains are linked by hydrogen bonds between molecules, resulting in a partly crystalline structure (Chundawat et al., 2011; Ling et al., 2022; Xu et al., 2013). Biomass crystallinity is a critical determinant of enzymatic hydrolysis of cellulose (Hall et al., 2010; Karimi & Taherzadeh, 2016; Ling et al., 2022). In this study, the CrI value of untreated biomass (59.5%) was lower than those observed for raw materials of wheat straw (69.6%) and hemp fiber (77.0%) (Xu et al., 2013). Pretreatment of various lignocellulosic biomasses with acids, alkalis, or other processes results in the partial elimination of amorphous components (hemicellulose and lignin) in the biomass, leading to a decrease in the nanocrystalline background on XRD and an increase in the CrI value (Hall et al., 2010; Ji et al., 2015; Premjet et al., 2022; Sathitsuksanoh et al., 2013; Yoo et al., 2020). These findings are consistent with the results of treatment of *V. pusilla* biomass with 70% and 75% H_3PO_4, and they revealed that these concentrations are incapable of decrystallizing cellulose. In addition, raising the CrI value of the pretreated biomass had no influence on the hydrolysis yields (Obeng et al., 2018; Premjet et al., 2022; Premjet et al., 2016) since the highest rates of GR and HEG were achieved after treatment with 75% H_3PO_4. The CrI value decreased

when the sample was treated with 80% H_3PO_4 because cellulose present in the treated biomass decomposed to a greater extent at higher concentrations. Moreover, during the regeneration of cellulose, the dissolved cellulose is reorganized, resulting in the modification and diminution of cellulose crystallinity (Hall et al., 2010; Obeng et al., 2018; Yoon et al., 2015; Zhang et al., 2006). In addition, at 85% H_3PO_4 concentration, the peak at 15.5° was missing, whereas the peak at 22.0° was displaced to a smaller angle, resulting in a broad asymmetrical peak with multiples at 12.0°, 19.9°, and 21.6°. At higher critical concentrations of H_3PO_4 (≥83%), high-order hydrogen bonds in crystalline cellulose fibers were destroyed to a greater degree, resulting in cellulose I being converted into cellulose II. This enables the cellulose fiber to decompose entirely (Sathitsuksanoh et al., 2013; Sathitsuksanoh et al., 2011; Sathitsuksanoh et al., 2012).

Ethanol Fermentation

To ascertain bioethanol production from weed biomass, the *V. pusilla* BH produced by enzymatic hydrolysis was fermented using yeast. Several authors reported that inhibitors are produced during the pretreatment of lignocellulose with acid, e. g., hydroxymethylfurfural (5-HMF), furfural, phenolics, and other aromatic compounds, which affect microbial fermentation (Arisht et al., 2019; Bhatia et al., 2020; Zhao et al., 2022). Compared to the ST medium, the non-detoxified BH medium had a greater influence on yeast cell proliferation than on ethanol production, as the BH medium may include molecules that restrict the growth of and ethanol production by *S. cerevisiae* TISTR 5339. Li et al., 2017 demonstrated that a series of inhibitors, including vanillin, phenol, syringaldehyde, 5-HMF, furfural, levulinic acid, acetic acid, and formic acid, affect the development of *S. cerevisiae* during glucose fermentation. These findings suggest that pretreatment of lignocellulosic materials with a high loading can result in higher sugar concentrations. However, this may also result in the development of a substantial amount of inhibitors. (Premjet et al., 2018b).

The ideal H_3PO_4 pretreatment conditions for fermentable sugar production were determined by calculating the mass balance of *V. pusilla* biomass to assess the overall process (Figure 9). The main components of the feedstock were glucan (48.1%), xylan (19.2%), Arabinan (1.2%), AIL (23.5%), and ASL (4.4%). After pretreatment with 75% H_3PO_4, approximately 51.1% of the pretreated material was recovered, including 37.6%

glucan, 2.5% xylan, 7.6% AIL, and 1.4% ASL. Both untreated and pretreated *V. pusilla* biomass were hydrolyzed with cellulase (30 FPU/g substrate) and β-glucosidase (60 U/g substrate). After 96 h of hydrolysis, the untreated sample had a HEG and GR of 19.4% and 115 g/1 kg biomass, respectively. However, the HEG and GR of the treated *V. pusilla* biomass were enhanced to 88.0% and 408 g/1 kg of biomass, respectively. Compared with the untreated sample, HEG and GR improved by approximately 4.5 and 3.5 times, respectively. Following the bioethanol production process, non-detoxified BH medium containing 20 g glucose/L prepared from the treated biomass of *V. pusilla* was fermented by *S. cerevisiae* TISTR 5339. As a result, approximately 51 g and 182 g of cellulosic ethanol were produced from glucose in untreated and pretreated biomass of *V. pusilla*, respectively. Compared to the untreated sample, bioethanol production of the treated *V. pusilla* biomass improved by approximately 3.5-fold. Our study demonstrates that non-detoxified BH medium generated from *V. pusilla* feedstock can be used as the primary source of carbon for cellulosic ethanol production by *S. cerevisiae* TISTR 5339.

Overall, our findings provide crucial information and suggest that H_3PO_4 treatment of *V. pusilla* feedstock has the potential to enhance the production of bioethanol. However, we suggest utilizing the response surface methodology as an experimental design in future studies to evaluate the impacts of significant pretreatment variables, including temperature, acid concentration, and duration, on solids recovery, HEG, and GR. To improve the ethanol yield by preventing inhibitory effects on organisms during fermentation, either the hydrolysate should be detoxified before being utilized as a substrate for bioethanol fermentation or organism-selected strains that are more resistant to inhibitors may be employed. This will streamline the process of producing cellulosic ethanol from weed biomass. However, microorganisms that can ferment carbohydrates and micronutrients present in the hydrolysate must be selected to obtain compounds with higher added value.

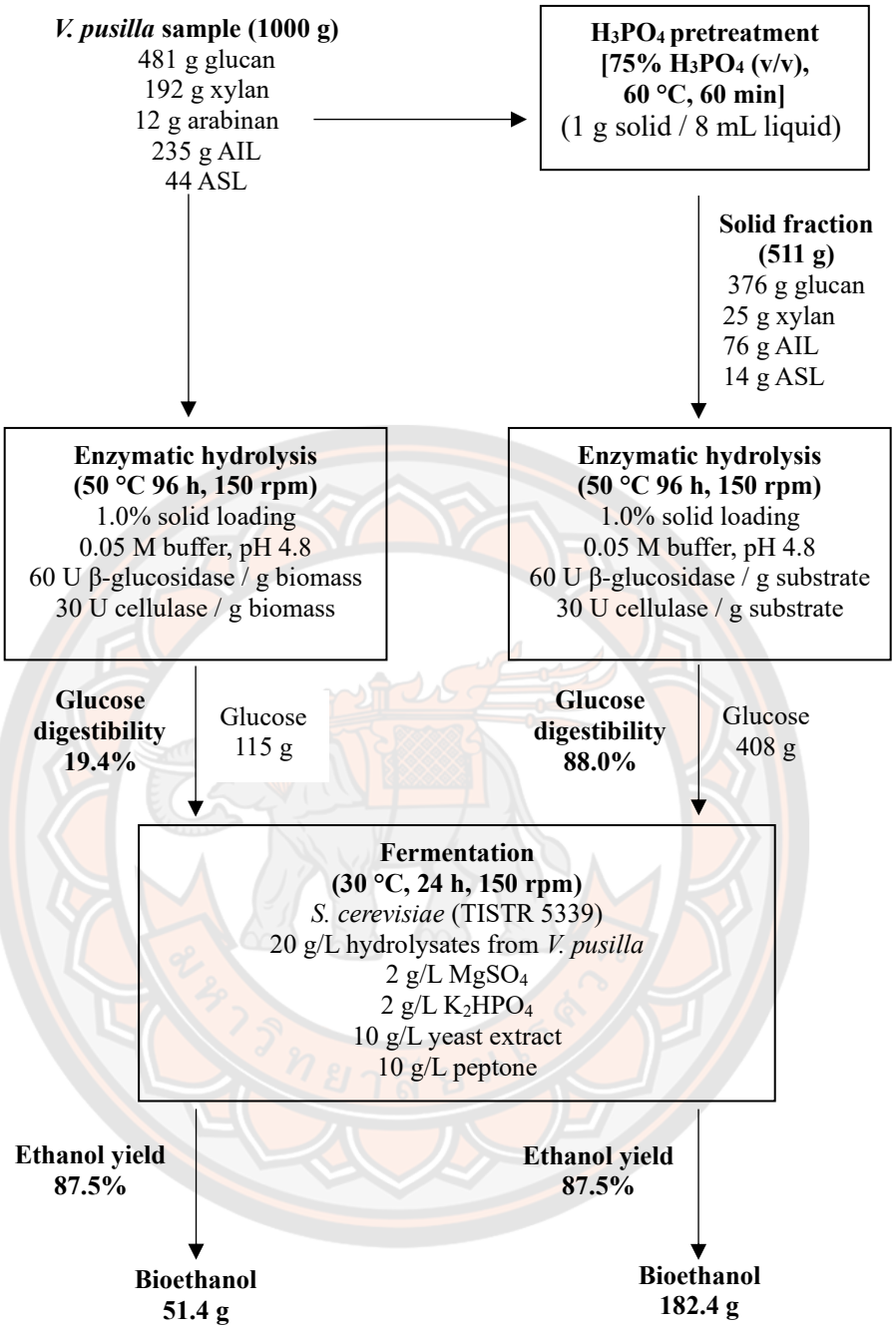

Figure 9 Mass balance of the *V. pusilla* feedstock for cellulosic ethanol production

Conclusions

The pretreatment of *V. pusilla* biomass with varying concentrations of H_3PO_4 under mild conditions was more efficient in eliminating xylan than lignin. The optimum pretreatment condition for the greatest improvement in GR and HEG was 75% H_3PO_4. Compared with the untreated sample, HEG and recovery GR improved by 4.5 and 3.5 times, respectively. The increase in both GR and HEG yields may be attributable to disintegration and dissolution of hemicellulose and lignin, which renders cellulose present in the treated biomass of *V. pusilla* vulnerable to cellulases. In addition, bioethanol fermentation in non-detoxified BH medium was established using treated *V. pusilla* biomass. Consequently, the ethanol yield was improved by approximately 3.5-fold compared with that of the untreated sample. Our research revealed that sugar platform-based biorefineries are able to utilize this sort of *V. pusilla* biomass to produce biofuels and other valuable compounds.

CHAPTER IV

PHYSICOCHEMICAL PRETREATMENT OF *VIETNAMOSASA PUSILLA* FOR BIOETHANOL AND XYLITOL PRODUCTION

Introduction

The depletion of nonrenewable fossil fuel reserves is impending, leading to a severe global energy crisis (Khan et al., 2021; Tusher et al., 2022). Consequently, the increasing demand for sustainable and renewable energy sources has driven extensive research into the production of renewable fuels and valuable compounds from nonedible biomass feedstocks (Casau et al., 2022; Khan et al., 2021; Tusher et al., 2022; Zhang et al., 2022). Lignocellulosic biomass is abundant, cost-effective, and may form part of an environmentally friendly conversion process (Sun et al., 2022). Extensive research has identified lignocellulose as an alternative energy source because of its high cellulose content, which makes it a suitable bioethanol production substrate (Lee et al., 2022; Messaoudi et al., 2022; Sun et al., 2022; Wongleang et al., 2023b).

Vietnamosasa pusilla, commonly known as Pai Pek (local Thai name), belongs to the Poaceae family and is a drought-resistant weed. *V. pusilla* has been classified as a "regionally controlled weed," indicating its status as a risk group for a specific region's primary crops and the environment (Pagad, 2016; Thuy et al., 2021). *V. pusilla* has been documented to be distributed in a geographic range that includes Cambodia, Laos, Thailand, and Vietnam. The major advantage of weed biomass is its rapid development despite harsh environmental conditions and minimal cultivation requirements (Obeng et al., 2019). However, the recalcitrant structure of all lignocellulosic materials, such as hemicellulose, lignin, and cellulose, makes digestion with enzymes difficult, leading to a lower bioconversion yield (Shukla et al., 2023; Zhang et al., 2022; Zhao et al., 2022). Therefore, pretreatment is crucial for the enhancement of sugar conversion and the facilitation of the release of fermentable sugars (Shukla et al., 2023; Zhao et al., 2022).

Among the various pretreatment methods, alkaline pretreatment has gained wide acceptance because of its simplicity, non-corrosiveness, lack of pollutants, and strong pretreatment effectiveness under mild reaction conditions, leading to fewer inhibitory compounds (An et al., 2021). This method creates an intricate lignin–hemicellulose network, enhancing lignin removal and increasing the surface area and porosity of the biomass. Consequently, enzymatic hydrolysis is enhanced, and a higher fraction of carbohydrates is preserved (Olatunji et al., 2022; Shukla et al., 2023; Zhao et al., 2022). NaOH pretreatment, which is a common alkaline pretreatment, effectively breaks down lignin by solubilizing lignin carbohydrate bonds, increasing the cellulose surface area, and reducing the sugar content (Shukla et al., 2023; Zhao et al., 2022). Furthermore, the effectiveness of the NaOH pretreatment can be improved by integrating a heating method that involves subjecting the material to high temperatures and pressures. NaOH-assisted thermal pretreatment is considered a highly efficient method for breaking down the recalcitrant structure of lignocellulose while minimally impacting cellulose and hemicellulose. This process effectively removes a large amount of lignin, reduces cellulose crystallinity, and enhances the specific surface area of cellulose for enzymatic saccharification (Obeng et al., 2021; Shukla et al., 2023; Zhao et al., 2022). This method has been successfully utilized for the pretreatment of several types of biomasses, including rice straw (Kumar et al., 2023), water hyacinth (Chinwatpaiboon et al., 2023), soybean hull (Sarmiento-Vásquez et al., 2022), cassava (Papathoti et al., 2021; Papathoti et al., 2022), *Arachis hypogea* shells (Olatunji et al., 2022), sugarcane bagasse (Melesse et al., 2022), *Pennisetum purpureum* (Haldar & Purkait, 2022; Manokhoon & Rangseesuriyachai, 2020), wheat straw (Wang et al., 2021), durian peel (Obeng et al., 2021), *Sicyos angulatus* (An et al., 2021), and *Miscanthus* × *giganteus* (Jung et al., 2020).

This study aimed to determine the effects of chemical pretreatment on *V. pusilla* feedstock. The *V. pusilla* enzymatic hydrolysate was used as a substrate for bioethanol and xylitol production. Our findings may help in optimizing the efficiency of cellulosic ethanol and xylitol production using plant-based biomass.

Materials and methods

Sample preparation

The above-ground biomass of *V. pusilla* was collected from a naturally occurring location in the Wang Thong district of Phitsanulok, Thailand. The specimen was subjected to a cleansing process using tap water and, following that, completed a drying period of five days under shaded conditions. Subsequently, the specimens were separated into shorter pieces of around 5 cm in length and subsequently subjected to pulverization through the utilization of a wood mill (Retsch, Haan, Germany). For subsequent examination and analysis, the powder was sieved through a 150–300 µm laboratory test sieve and stored in bottles at 25 °C (Hames et al., 2008).

Chemical composition analysis

The details of these analytical methodologies are described in a previous report by Premjet et al., 2018a. The composition of *V. pusilla* feedstock was investigated before and after alkali pretreatment. National renewable energy laboratory techniques were employed to evaluate the sugar and lignin contents of the sample (Sluiter et al., 2012). Ultraviolet–visible (UV-Vis) spectrophotometer (Analytik Jena Specord 40, Analytik Jena AG, Jena, Germany) absorbance at 205 nm was utilized to assess acid-soluble lignin.

Monomeric sugar, ethanol, and xylitol were analyzed using a high-performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies, Waldbronn, Germany) system integrated with a refractive index detector (G1362A; Agilent Technologies, Waldbronn, Germany) and a Bio-Rad Aminex-HPX 87H column (300 mm × 7.8 mm; Hercules, CA, USA), which were maintained at 55 and 60 °C, respectively. The mobile phase consisted of 0.005 M sulfuric acid with a 0.6 mL/min flow rate. The injection volume was 20 µL (Premjet et al., 2022).

Alkaline-Catalyzed Steam Pretreatment

The pretreatment technique was employed in a previous study conducted by Obeng et al., 2019. In a 125-mL Erlenmeyer flask, 3 g of *V. pusilla* feedstock was soaked in 24 mL of NaOH solution (1%, 2%, 3%, and 4% w/v), and the flasks were autoclaved (Hirayama, HVA-85, Toyono-cho, Kasukabe-shi, Saitama, Japan) at 120 °C

and 15 psi of pressure for 60 min. Then, the *V. pusilla* sample with the ideal NaOH concentration was subjected to treatment at 110 °C, 120 °C, and 130 °C at 15 psi of pressure for 60 min. At the end of the pretreatment process, the slurry was rapidly ice-cooled and then filtered. The solid portion was washed thoroughly with deionized water to attain a neutral pH. The washed alkaline-catalyzed steam pretreated biomass samples were used for further analysis (Obeng et al., 2019).

Enzymatic Hydrolysis

The process of enzymatic hydrolysis of both raw and treated *V. pusilla* biomass was monitored using methods that have been previously reported with slight modifications (Obeng et al., 2019). A 50 mL Erlenmeyer flask containing 10 mL of digestion solution was used to hydrolyze 0.1 g of biomass (dry weight) from both the raw and pretreated samples. The enzyme mixture used in the digestion process included 30 filter paper units (FPU) of cellulase (*Trichoderma reesei* C2730, Sigma-Aldrich, St. Louis, MO, USA), 60 U xylanase (*Trichoderma longibrachiatum* X2629, Sigma-Aldrich, Co., St. Louis, USA), and 60 U-glucosidase (Oriental Yeast Co., Ltd., Tokyo, Japan) per gram of dry biomass in a sodium citrate buffer (pH 4.8) with 2% (w/v) sodium azide. The flask of each reaction was incubated at 50 °C and 150 rpm for 96 h in a rotary shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA). At 12, 24, 48, 72, and 96 h intervals, 200 µL of hydrolysate were collected for sugar determination (Obeng et al., 2019).

The hydrolysis efficiencies of glucose (HEG) and xylose (HEX), glucose recovery (GR), and xylose recovery (XR) were determined using the following equations from Obeng et al. (Obeng et al., 2019) and Sun et al. (Sun et al., 2022):

$$\text{HEG (\%)} = \frac{\text{Glucose generated by hydrolysis (g)} \times 100}{1.11 * \text{Initial glucan in substrate (g)}} \quad (7)$$

$$\text{HEX (\%)} = \frac{\text{Xylose generated by hydrolysis (g)} \times 100}{1.14 * \text{Initial amount of xylan in substrate (g)}} \quad (8)$$

$$\text{GR (\%)} = (\text{solid recovery} \times \text{glucan in substrate} \times 1.11 \times \text{HEG}) \times 100 \quad (9)$$

$$\text{XR (\%)} = (\text{solid recovery} \times \text{xylan in substrate} \times 1.14 \times \text{HEX}) \times 100 \quad (10)$$

Where 1.11 and 1.14 are the conversion factors for glucan and xylan to glucose and xylose, respectively.

Determination of Biomass Microstructure

The specific method for determination is described in a previous study (Obeng et al., 2019; Obeng et al., 2021). The raw and treated *V. pusilla* feedstocks were freeze-dried. All samples were gold-coated and mounted on aluminum stubs. All coated biomasses were captured employing field emission scanning electron microscope (FESEM; Thermo Fisher Scientific, Apero S, Waltham, MA, USA) (Obeng et al., 2019; Obeng et al., 2021).

Crystallinity of Biomass

The method of analysis was previously explained in a study by (Obeng et al., 2019; Obeng et al., 2021). Both the untreated and treated feedstocks were washed three times with acetone and left at 25 °C for 12 h. They were then ground and sieved, and fraction-size screens ranging from 100 to 150 μm were collected. X-ray diffraction (PANalytical X'pert Pro, PW 3040/60, Almelo, The Netherlands) was used to measure the crystallinity index (CrI) of all samples. The samples were scanned between 10°–40° at 0.02° s^{-1}/min.

Microorganisms

In this study, bioethanol and xylitol were produced using *Saccharomyces cerevisiae* TISTR 5339 and *Candida tropicalis* TISTR 5171, respectively. These yeast strains were purchased in special order and submitted to the Thailand Institute of Science and Technology (TISTR).

Preparation of Seed Culture

A single loop from yeast strain, *S. cerevisiae* TISTR 5339 and *C. tropicalis* TISTR 5171, was used as the initial inoculum. *S. cerevisiae* TISTR 5339 was placed into a 50 mL Falcon tube with 10 mL of yeast malt medium and incubated with a rotating shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA) at 30 °C for 18 h. *C. tropicalis* TISTR 5171 was transferred to a 50 mL Falcon tube containing 10 mL of yeast malt peptone medium and incubated using a rotary shaker at 30 °C for 24 h.

Preparing Biomass Hydrolysate for Ethanol Production

The biomass hydrolysate (BH) was prepared according to procedures outlined in a previous study (Premjet et al., 2018b). The BH of *V. pusilla* obtained from enzymatic hydrolysis was boiled for 20 minutes at 100 ℃ in a water bath to terminate the reaction. The hydrolysate was centrifuged for 2 h at 12,000× g. Afterward, it was passed through a glass microfiber filter to eliminate impurities. Then, a rotary evaporator was used to concentrate the hydrolysate until it contained approximately 20 g/L glucose. The hydrolysate was neutralized with 1 M NaOH to increase the pH of hydrolysate to 6. Finally, the BH of *V. pusilla* was maintained at 4 ℃ for future experiments.

Preparing Biomass Hydrolysate for Xylitol Production

The liquid fraction, a byproduct of ethanol production, was separated by centrifugation at 12,000× *g* for 1 h and passed through a glass microfiber filter to remove yeast cells and other contaminants. Subsequently, a rotary vacuum evaporator containing 20 g/L xylose was used to condense the liquid into xylose biomass hydrolysate (XBH). The pH of XBH was adjusted to 5.5 and stored overnight at 4 °C.

Then, the mixture was centrifuged for 1 h at 12,000× *g* and passed through a glass filter. The XBH was then stored at 4 °C for use in future studies (Premjet et al., 2018b).

Medium for Bioethanol and Xylitol Production

Ethanol was produced using the control and the BH media. The BH medium contained glucose derived from the biomass, whereas commercial glucose was used as the carbon source in the control medium. Each medium was prepared by adding 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 2 g/L $MgSO_4$, and 2 g/L K_2HPO_4, and the pH was set at 6 (Premjet et al., 2018b).

Xylitol production requires XBH and control media. The XBH medium used xylose derived from biomass as its carbon source, whereas the control medium relied on commercial xylose. Each medium contained 20 g/L xylose, 5 g/L yeast extract, 1 g/L $MgSO_4$, 1 g/L KH_2PO_4, and 1 g/L NH_4Cl at a pH of 5.5 (Ngamsirisomsakul et al., 2021).

In this study, all liquid culture media were prepared under aseptic conditions and passed through a Millipore filter with a 0.2 μm pore size (Merck Ltd. Tullagreen, Carrigtwohill, Co., Cork, Ireland).

Ethanol Fermentation

The production of bioethanol utilized 50 mL of both BH and a control medium. Both media were inoculated with 2% (v/v) *S. cerevisiae* TISTR 5339 seed culture and incubated at 30 °C on a rotary shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA) at 150 rpm for 24 h. After 3, 6, 9, 12, 15, 18, 21, and 24 h of incubation, the liquid fraction was harvested for HPLC measurement of sugar uptake and ethanol generation. The optical density (OD) at 600 nm was utilized to monitor the growth of microorganisms using a UV spectrophotometer (Premjet et al., 2018b).

Xylitol Fermentation

The total volume of the XBH and control media used for xylitol production was 50 mL. A 5% seed culture of *C. tropicalis* TISTR 5171 was injected into both media and then incubated on a rotary shaker (Innova 4340, New Brunswick Scientific Company, Edison, New Jersey, USA) at 200 rpm for 7 days at 30 °C. The amounts of

xylose consumed, and xylitol produced, were subsequently determined by collecting 1 mL of the liquid fraction daily for HPLC analysis. The xylitol yield was determined using the following equation:

$$\text{Xylitol yield (\%)} = \frac{\text{Xylitol in fermentation liquid (g)} \times 100}{0.905 \times \text{Xylose initial (g)}} \quad (11)$$

Results

Composition of Raw Biomass

The chemical structure of the *V. pusilla* biomass (dry weight) sample comprised glucan (48.1 ± 0.3%), xylan (19.2 ± 0.4%), arabinan (1.2 ± 0.1%), acid insoluble lignin (AIL: 23.5 ± 0.1%), and acid soluble lignin (ASL: 4.4 ± 0.1%). These components constituted approximately 68.5% and 27.9% of the total carbohydrate and lignin contents, respectively. In a previous chapter, this composition was evaluated and discussed.

Effect of NaOH Concentration on Composition

V. pusilla feedstock was pretreated with 1%, 2%, 3%, and 4% NaOH at 120 °C for 60 min to determine the optimal NaOH concentration for pretreatment. Changes in the chemical composition of the biomass before and after pretreatment with varying concentrations of NaOH are presented in Table 7. This study revealed that NaOH pretreatment significantly influences the partial dissolution of lignin and carbohydrates from this weed biomass.

As the concentration of NaOH increased, both glucan and xylan contents increased significantly ($p < 0.05$). The relative glucan content gradually increased by 55.3 ± 0.5%, 67.2 ± 0.1%, 68.0 ± 0.0%, and 71.0 ± 0.2%, when treated with 1%, 2%, 3%, and 4% NaOH, respectively. The xylan content increased by 21.9 ± 0.2% and 21.3 ± 0.3% after treatment with 1% and 2% NaOH, respectively, although the improvements were not statistically significant ($p < 0.05$). When the concentration of NaOH increased to 3% and 4%, the xylan content declined substantially to 20.1 ± 0.2% and 17.8 ± 0.4%, respectively. Arabinan was not detected in any treated samples (Table 7).

Table 7 Chemical compositions of *V. pusilla* after NaOH pretreatment at 120 °C

Composition (% dw)	Untreated Sample	Concentration of NaOH			
		1%	2%	3%	4%
Glucan	48.1±0.3 [e]	55.3±0.5 [d]	67.2±0.1 [c]	68.0±0.0 [b]	71.0±0.2 [a]
Xylan	19.2±0.4 [c]	21.9±0.2 [a]	21.3±0.3 [a]	20.1±0.2 [b]	17.8±0.4 [d]
Arabinan	1.2±0.1	n.d.	n.d.	n.d.	n.d.
AIL*	23.5±0.1 [a]	21.8±0.0 [b]	20.9±0.1 [c]	18.1±0.5 [d]	15.4±0.3 [e]
ASL**	4.4±0.1 [a]	3.0±0.0 [b]	2.9±0.0 [b]	2.9±0.0 [b]	2.9±0.0 [b]
Total lignin	27.9±0.2 [a]	24.8±0.1 [b]	23.9±0.1 [c]	21.0±0.5 [d]	18.3±0.3 [e]

Note: AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin

The superscripted characters within rows indicate the statistical significance of the differences between them ($p < 0.05$), n.d. = not determined

In addition, increasing the NaOH concentration from 1% to 4% resulted in substantial declines in AIL and total lignin content. The minimum quantities of AIL (15.4 ± 0.3%) and the total lignin (18.3 ± 0.3%) were obtained by feedstock treated with 4% NaOH. However, ASL content significantly decreased ($p < 0.05$) with 1% NaOH, whereas higher concentrations of NaOH (2%–4%) had no effect on ASL reduction (Table 7). The results reveal that the total lignin content declined from 27.9 ± 0.2% (raw biomass) to 18.3 ± 0.3%. Approximately 66% of delignification was attributed to this pretreatment method (Figure 10).

The results reveal a gradual reduction in the recovery yields of solids, xylan and glucan, which progressively declined as the NaOH concentration raised (Figure 11). After treating the feedstock with 1%–4% NaOH, the solid recovery yields ranged from 51.4 ± 0.8% to 65.5 ± 0.7%, whereas the xylan recovery yields ranged from 47.5 ± 1.0% to 74.7 ± 0.6%. However, there was no significant ($p < 0.05$) difference in the glucan recovery yield after pretreatment with 1%, 3%, or 4% NaOH. NaOH concentrations of 2% resulted in the highest glucan recovery yields (81.0 ± 0.1%). The results indicate that, by pretreating the sample with 1%–4% NaOH, solid and glucan recovery yields greater than 50% and 75% were achieved, respectively. Nevertheless, a xylan recovery yield greater than 50% was obtained by treatment with 1%–3% NaOH.

The maximum yields for solid recovery (65.5 ± 0.7%) and xylan recovery (74.7 ± 0.6%) were achieved with 1% NaOH, whereas the highest yield for glucan recovery (81.0 ± 0.1%) was acquired with 2% NaOH.

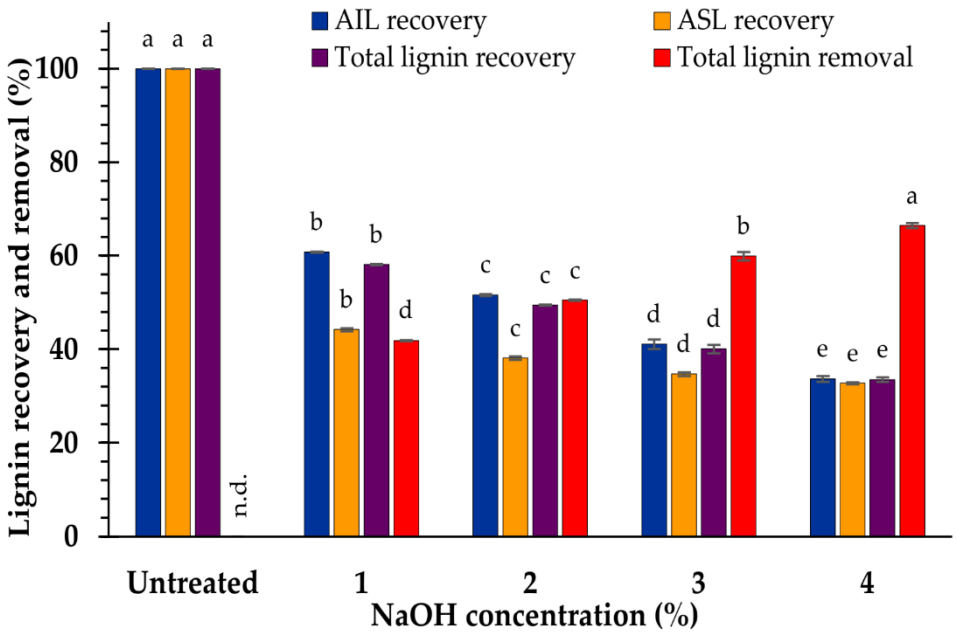

Figure 10 Lignin recovery and removal after pretreatment at difference NaOH concentration

Note: The significance of the variations in concentrations ($p < 0.05$), is shown by the superscripted characters; n.d. = not determined.

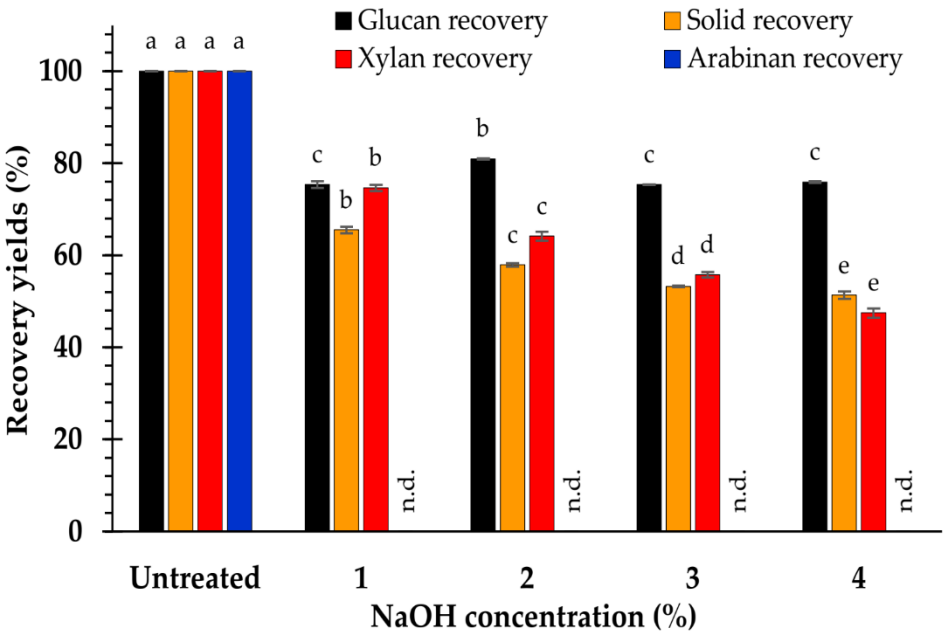

Figure 11 Recovery yields after pretreatment at difference NaOH concentration

Note: The significance of the variations in concentrations ($p < 0.05$), is shown by the superscripted characters; n.d. = not determined.

Effect of NaOH Concentration on Hydrolysis Yield

To determine the suitable NaOH concentration for the pretreatment of weedy feedstock, the treated biomass samples were subjected to enzymatic hydrolysis. The highest yields of GR, HEG, XR, and HEX were obtained after 96 h of hydrolysis (Figures 12 and 13). The findings reveal that the yields of untreated feedstock for GR, HEG, XR, and HEX were 11.5 ± 0.0%, 19.4 ± 0.0%, 5.0 ± 0.0%, and 20.1 ± 0.1%, respectively. After treating the feedstock with 1%, 2%, 3%, and 4% NaOH, the yields of GR, HEG, XR, and HEX were significantly ($p < 0.05$) higher than those of the untreated material. However, the increase in HEG yields of 2% (88.4 ± 0.0%), 3% (87.8 ± 0.2%), and 4% NaOH (87.7 ± 0.3%) did not differ statistically. Nevertheless, the greatest yield of GR (42.4 ± 0.0%) was obtained from a sample treated with 2% NaOH.

Moreover, the levels of GR diminished at NaOH concentrations of 3% (39.2 ± 0.1%) and 4% (39.5 ± 0.1%), but no significant differences were observed ($p < 0.05$). The highest yield of HEX (85.3 ± 0.4%) was generated from the sample treated with 4% NaOH, whereas the yield of XR (10.1 ± 0.0%) was the lowest. However, the XR

yields were enhanced at 1% (11.9 ± 0.1), 2% (11.8 ± 0.1), and 3% (12.1 ± 0.1) NaOH, but the difference was not statistically significant between them (Figures 12 and 13). Additionally, treatment of *V. pusilla* biomass with 2% NaOH at 120 °C for 60 min resulted in 3.7- and 2.4-fold boosts in GR and XR yields, respectively, when compared with untreated feedstock. The yields of HEG and HEX increased by approximately 4.6 and 3.7 times, respectively.

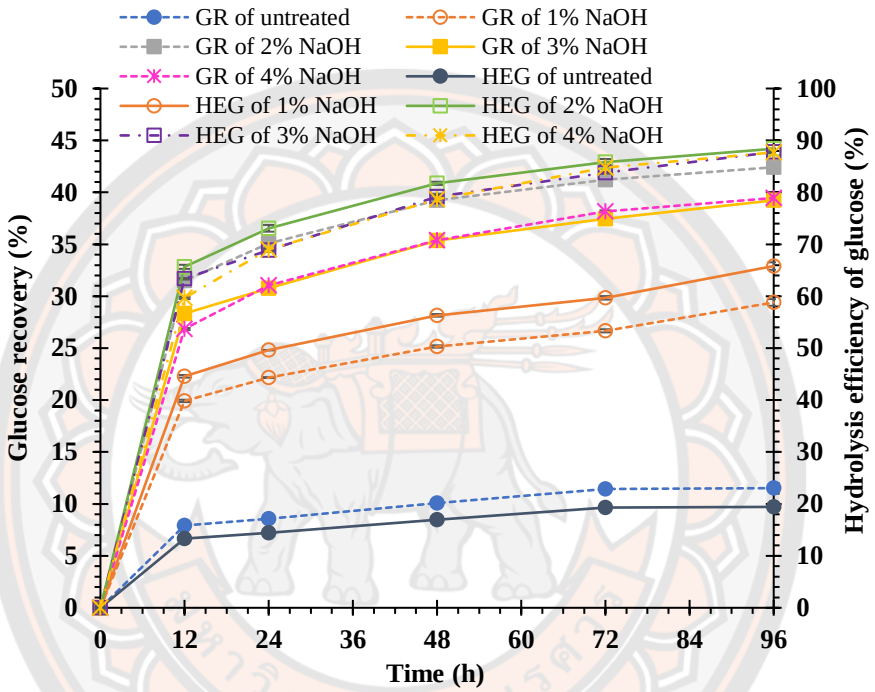

Figure 12 Glucose recovery (GR) and hydrolysis efficiency of glucose (HEG) of untreated and pretreated biomass of *V. pusilla*

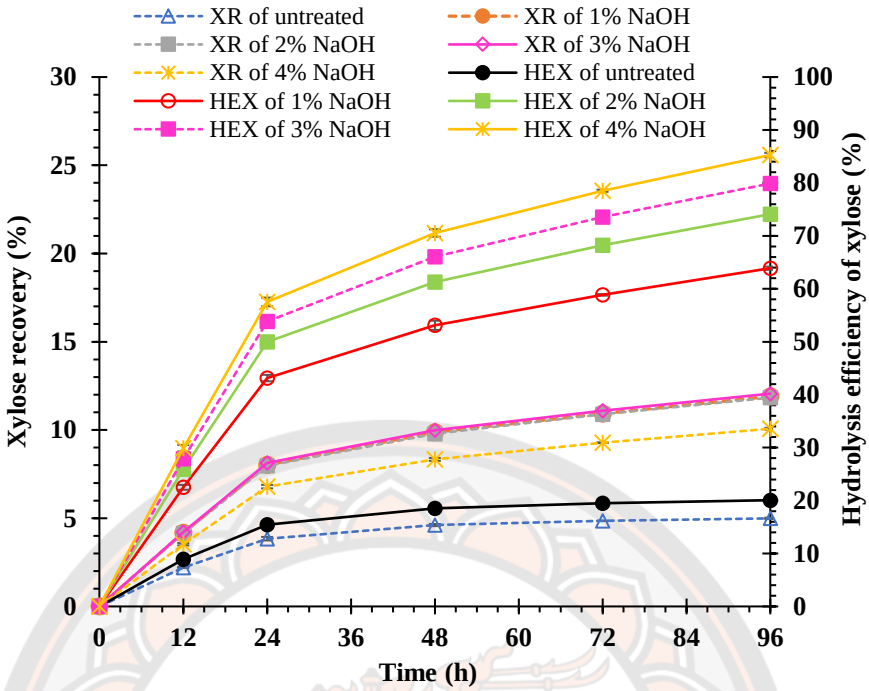

Figure 13 Xylose recovery (XR) and hydrolysis efficiency of xylose (HEX) of untreated and pretreated biomass of *V. pusilla*

Effect of Temperature on Biomass Composition

To determine the suitable pretreatment temperature, *V. pusilla* feedstock was treated with 2% NaOH for 60 min at various temperatures. The relative glucan content increased to 65.1 ± 0.3%, 67.2 ± 0.1%, and 67.1 ± 0.3% when the temperature was raised to 110 °C, 120 °C, and 130 °C, respectively. The increase in glucan content at 120 °C and 130 °C was not statistically different ($p < 0.05$). However, the maximum relative xylan content (22.5 ± 0.1%) was observed at 110 °C, which was subsequently reduced to 21.3 ± 0.3% and 19.3 ± 0.2% at 120 °C and 130 °C, respectively. Arabinan did not appear in any of the treatment materials (Table 8).

Table 8 Chemical compositions of *V. pusilla* after 2% NaOH pretreatment at different temperatures

Composition (% dw)	Untreated Sample	Temperatures (°C)		
		110	120	130
Glucan	48.1±0.3 ^{c}	65.1±0.3 ^{b}	67.2±0.1 ^{a}	67.1±0.3 ^{a}
Xylan	19.2±0.4 ^{c}	22.5±0.1 ^{a}	21.3±0.3 ^{b}	19.3±0.2 ^{c}
Arabinan	1.2±0.1	n.d.	n.d.	n.d.
AIL*	23.5±0.1 ^{a}	23.0±0.1 ^{a}	20.9±0.1 ^{b}	16.1±0.5 ^{c}
ASL**	4.4±0.1 ^{a}	3.2±0.0 ^{b}	2.9±0.0 ^{c}	2.9±0.0 ^{c}
Total lignin	27.9±0.2 ^{a}	26.1±0.1 ^{b}	23.9±0.1 ^{c}	19.0±0.5 ^{d}

Note: AIL* = acid-insoluble lignin, ASL** = acid-soluble lignin

The superscripted characters within rows indicate the statistical significance of the differences between them ($p < 0.05$), n.d. = not determined

At 110 °C, a slight variation in AIL content between the treated (23.0 ± 0.1%) and untreated samples (23.5 ± 0.1%) was observed. The AIL content was then substantially reduced at higher temperatures (120 °C and 130 °C). Moreover, a decrease in ASL was observed when the temperature increased. The amount of ASL diminished at 120 °C (2.9 ± 0.0%) and 130 °C (2.9 ± 0.0%) and did not differ significantly ($p < 0.05$). Nevertheless, a gradual decrease in the total lignin content coupled with an increase in the pretreatment temperature was observed. Feedstock treated at 130 °C

resulted in the smallest amounts of AIL (16.1 ± 0.5%), ASL (2.9 ± 0.0%), and total lignin (19.0 ± 0.5%). The evidence showed that the overall amount of lignin decreased from 27.9 ± 0.2 (untreated sample) to 19.0 ± 0.5% (at 130 °C) (Table 8). This pretreatment strategy was responsible for approximately 66% delignification, which was equivalent to that of the sample treated with 4% NaOH, as shown in Figures 10 and 14.

The results reveal that the temperature increase accompanied a reduction in the solid, xylan, and glucan recovery yields. At 110 °C, the highest recovery yields were obtained for solids (65.4 ± 0.6%), glucan (88.6 ± 0.4%), and xylan (76.6 ± 0.4%). However, the minimum amounts of the solid (49.9 ± 0.7%), xylan (50.1 ± 0.6%), and glucan (69.7 ± 0.3%) recovery yields were obtained at 130 °C. Therefore, the temperature may significantly influence the decline in the recovery yields of solids, xylan, glucan, and total lignin (Figure 15).

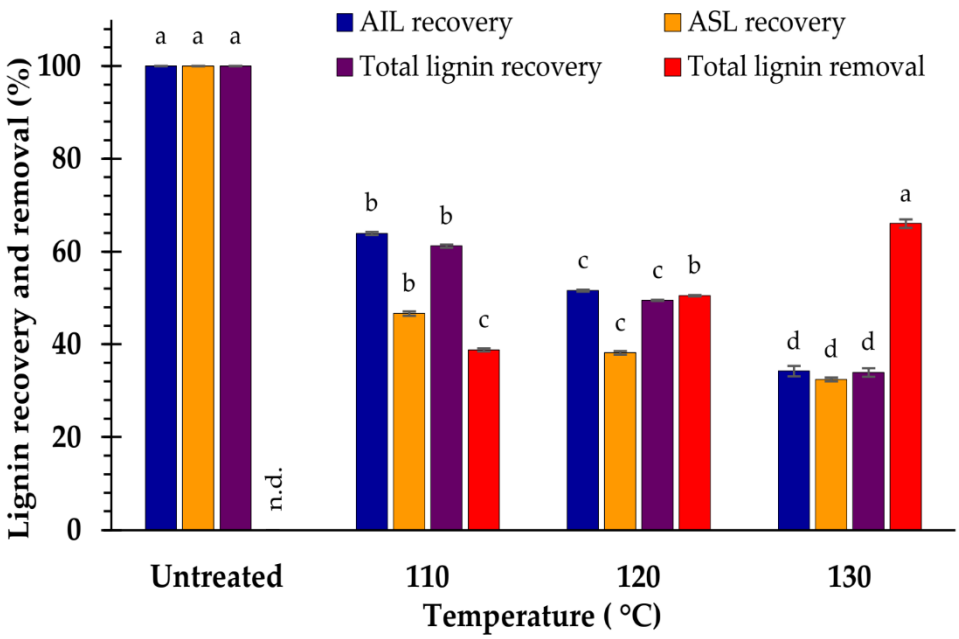

Figure 14 Lignin recovery and removal after pretreatment at different temperatures

Note: The significance of the variations in temperature ($p < 0.05$), is shown by the superscripted characters; n.d. = not determined

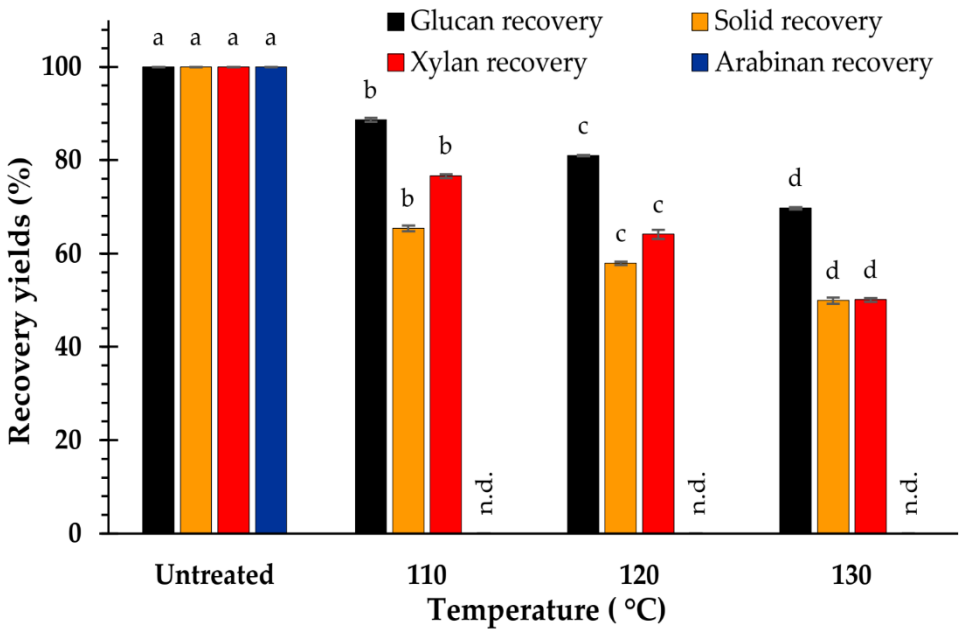

Figure 15 Recovery yields after pretreatment at different temperatures

Note: The significance of the variations in temperature ($p < 0.05$), is shown by the superscripted characters; n.d. = not determined

Effect of Temperature on Hydrolysis Yield

To determine the optimal pretreatment temperature, the biomass of *V. pusilla* was treated with 2% NaOH at various temperatures and subjected to enzymatic hydrolysis. Pretreatment of the feedstock with 2% NaOH at 110 °C, 120 °C, and 130 °C was demonstrated to considerably increase the quantities of GR, HEG, XR, and HEX (Figures 16 and 17). Compared with the untreated sample, the GR yields expanded to 37.6 ± 0.1%, 42.4 ± 0.0%, and 36.9 ± 0.1% at 110 °C, 120 °C, and 130 °C, respectively. When the temperature increased, the HEG and HEX yields increased steadily. The highest yields of HEG (89.3 ± 0.3%) and HEX (84.4 ± 0.2%) were achieved at 130 °C. Whereas the highest yield of XR (12.9 ± 0.0%) was achieved at 110 °C. The yields dropped dramatically at higher temperatures (120 °C and 130 °C). These findings confirm that samples treated with 2% NaOH at 120 °C for 60 min produced the highest yield of GR (42.4 ± 0.0%).

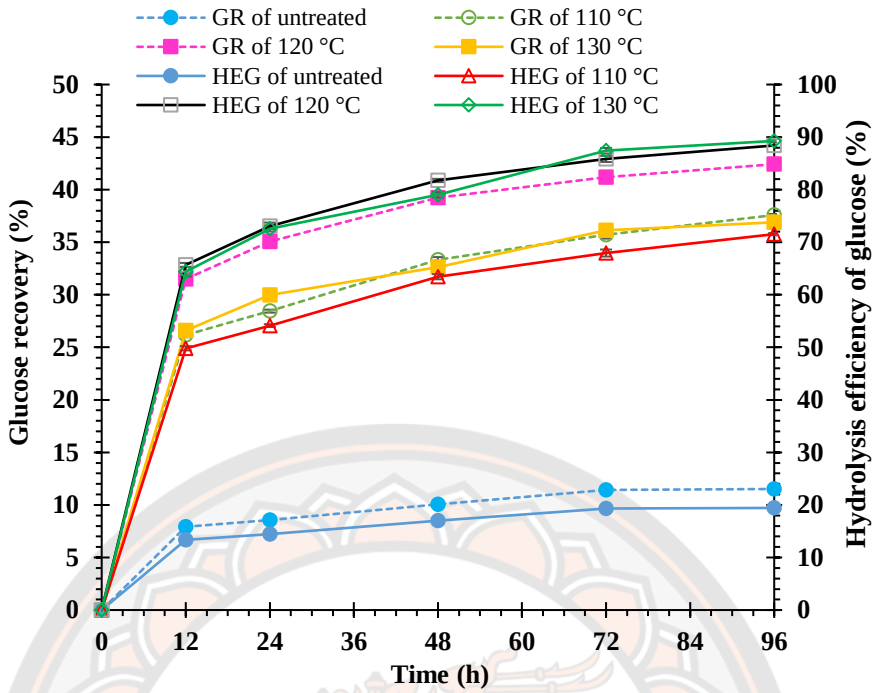

Figure 16 Glucose recovery (GR) and hydrolysis efficiency of glucose (HEG) of untreated and pretreated biomass of *V. pusilla*

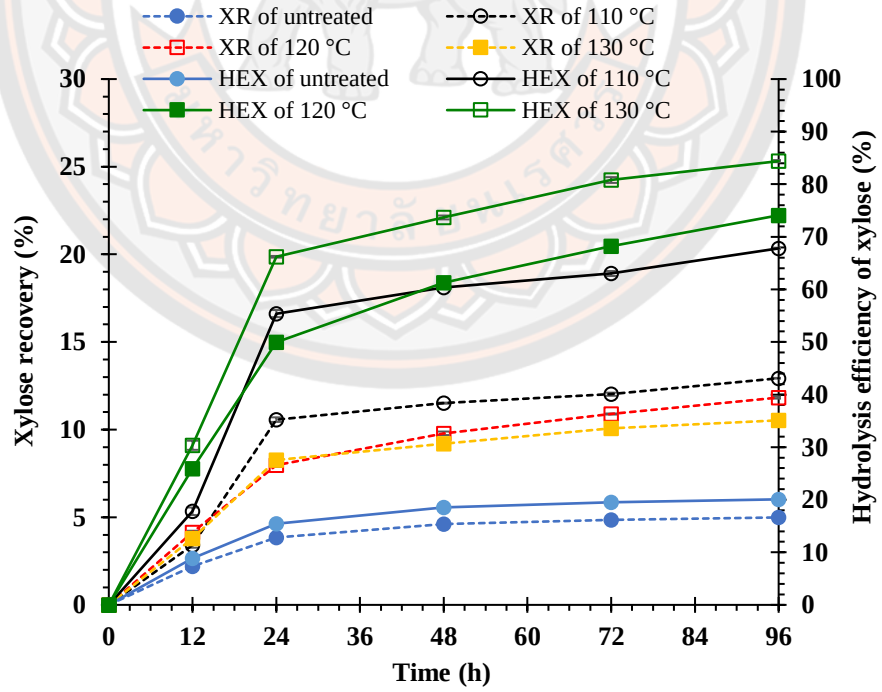

Figure 17 Xylose recovery (XR) and hydrolysis efficiency of xylose (HEX) of untreated and pretreated biomass of *V. pusilla*

Effect of Pretreatment on Crystalline Structure

The effect of NaOH pretreatment on *V. pusilla* crystalline cellulose was investigated using XRD. Consequently, all the untreated and treated biomass samples with varying NaOH concentrations and temperatures were analyzed. The XRD patterns are shown in Figures 18 and 19. All the treated and untreated samples were identified as cellulose I based on their XRD patterns, which consisted of two significant peaks at 2θ = 15.5° and 2θ = 22°. The untreated sample exhibited the lowest CrI value (59.5%). The CrI values of all treated samples increased after pretreatment with different NaOH concentrations and temperatures applied to the feedstock. The sample treated with 1%–4% NaOH exhibited increased CrI values ranging from 66.2% to 67.9%. However, when this sample was treated at various temperatures, the CrI increased from 67.4% to 68.5% (Table 9).

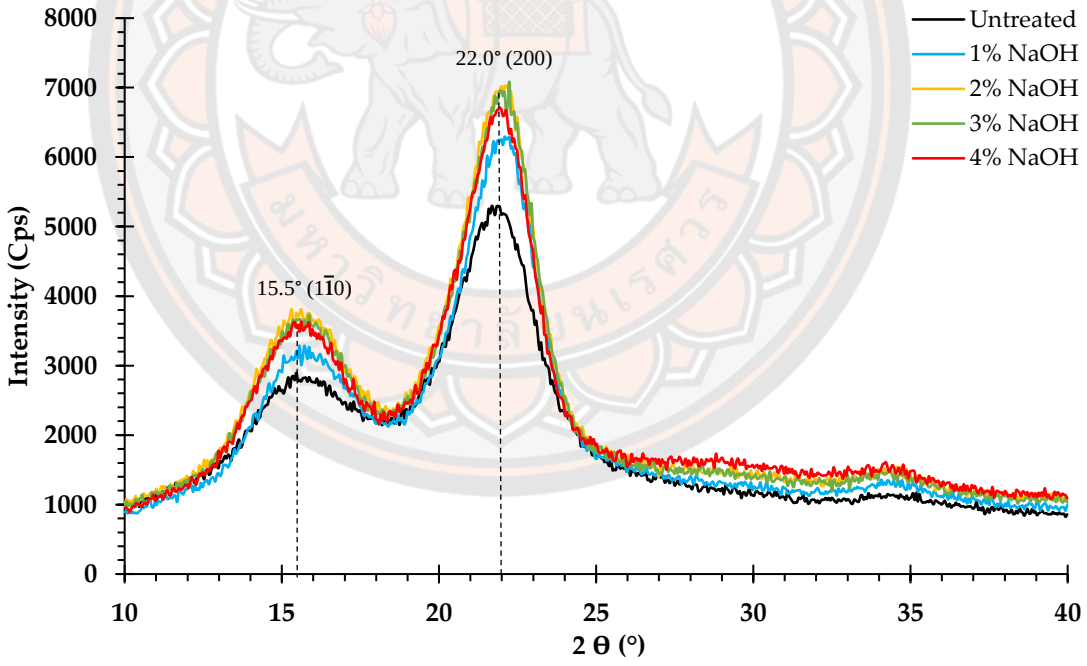

Figure 18 X-ray diffraction pattern of untreated and pretreated *V. pusilla* with varying concentrations of NaOH

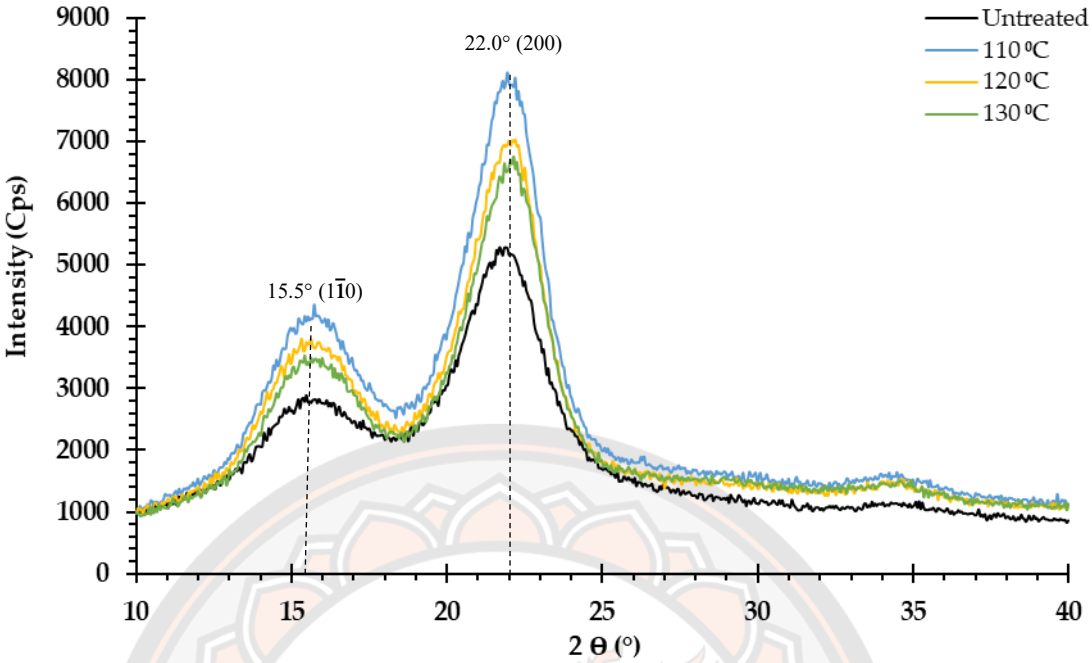

Figure 19 X-ray diffraction pattern of untreated and 2% NaOH treated *V. pusilla* with varying temperatures

Table 9 Crystallinity index of the untreated and NaOH-pretreated of *V. pusilla*

Concentration (%)	Temperature (°C)	CrI (%)
Untreated	-	59.5
1% NaOH	120	66.2
2% NaOH	120	67.6
3% NaOH	120	67.7
4% NaOH	120	67.9
2% NaOH	110	68.5
2% NaOH	130	67.4

Effect of Pretreatment on Morphological Structure

SEM was used to evaluate the surface morphology of untreated *V. pusilla*, which was subjected to alkaline-assisted thermal pretreatment with varying concentrations of NaOH and temperatures. The results are shown in Figures 20A–E and 21A–D. The untreated specimens exhibited a dense morphology characterized by bundled fiber organization and a smooth surface devoid of porosity. Following

exposure to different concentrations of NaOH and temperatures, the surface morphology of the sample was considerably altered, resulting in the disorganization of the fibers. Furthermore, an increase in NaOH concentration and temperature resulted in a higher degree of surface fragmentation, which was characterized by the presence of distinct elongated fissures, deep crevices, and pores (Figures 20A–E and 21A–D).

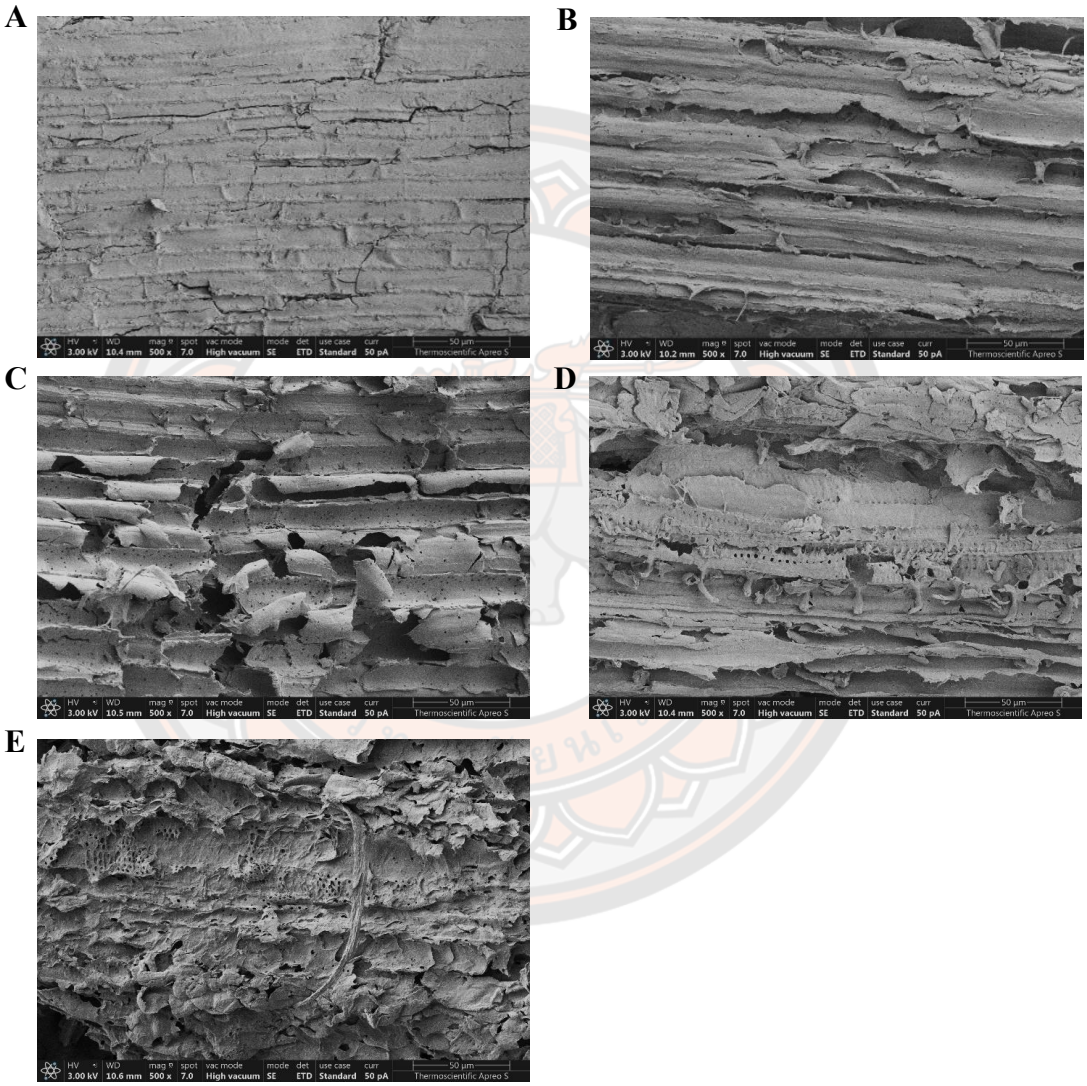

Figure 20 Surface morphology of (A) untreated and NaOH-pretreated samples at (B) 1%, (C) 2%, (D) 3%, and (E) 4% NaOH

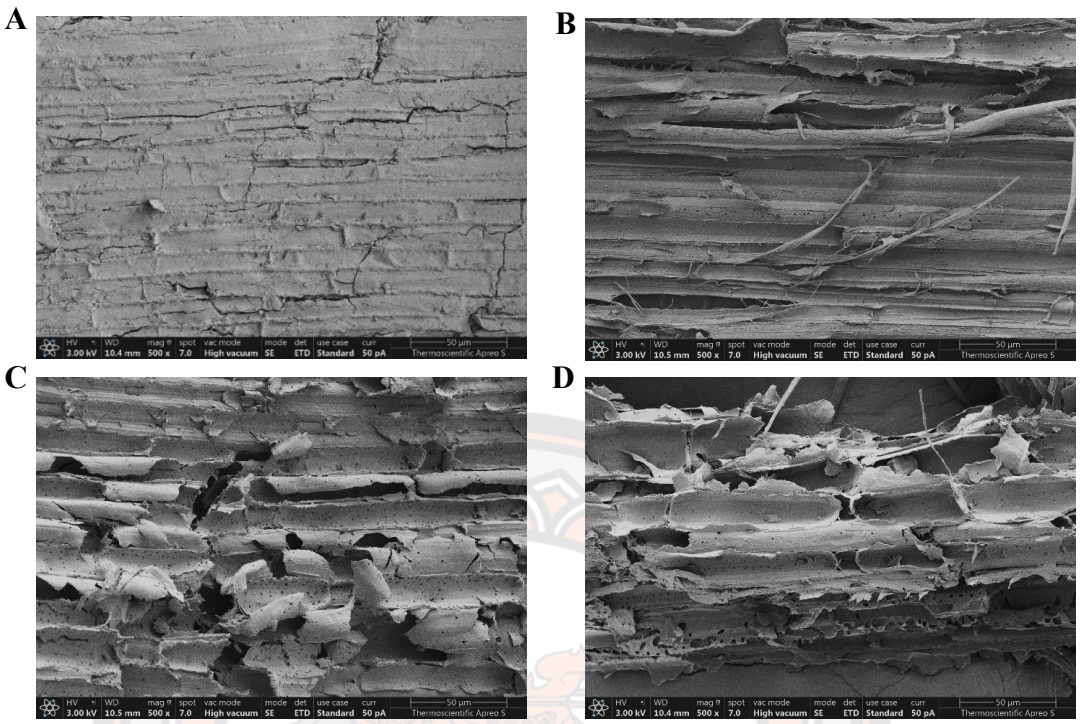

Figure 21 Surface morphology of (A) untreated and 2% NaOH-pretreated samples at (B) 110 °C, (C) 120 °C, and (D) 130 °C

Ethanol Fermentation

Enzymatic hydrolysis produces a sugar solution called biomass hydrolysate (BH), which contains glucose and xylose. To utilize the glucose in *V. pusilla* BH for ethanol fermentation, the BH was concentrated using a rotary vacuum evaporator to generate approximately 20 g/L glucose. Upon completion of the concentration procedure, BH was found to contain a mixture of 20 and 10 g/L of glucose and xylose, respectively. These findings reveal that xylose, a byproduct of BH, has great potential for utilization in the production of other high-value products. To obtain the maximum benefit from *V. pusilla* BH medium, we first fermented the medium without detoxification using *S. cerevisiae* TISTR 5339 to create bioethanol. Within 24 h, we analyzed the consumption of glucose and xylose, amount of bioethanol, exponential growth of yeast cells, and pH of the media to evaluate the potential of *V. pusilla* BH medium for bioethanol production. The standard medium contained 20 g/L of D-glucose (98% reagent grade) and 10 g/L of xylose (99% reagent grade). The results are summarized in Figures 22 and 23. The yeast strain *S. cerevisiae* TISTR 5339 rapidly

consumes glucose and concurrently produces ethanol. In both standard and BH media, glucose was consumed entirely within 9 h. After 15 h of incubation, the standard medium generated a maximal ethanol yield of 91.8 ± 1.6% or 9.7 ± 0.2 g/L, whereas the BH medium allowed a yield of 89.6 ± 0.1% or 9.4 ± 0.0 g/L. After incubation for 15 h, ethanol production in both media decreased slightly. Addition-ally, xylose in both media was slightly diminished, but it was still present in the standard (7.9 ± 0.2 g/L) and the BH media (7.1 ± 0.5 g/L), as shown in Figure 22.

The growth patterns of *S. cerevisiae* TISTR 5339 were similar in both media, and the pH changes were also comparable. After 24 h of incubation, the yeast growth rate in the standard medium reached its maximum value. However, the highest growth rate of yeast cells was observed after 12 h of incubation in BH medium, with the growth rate decreasing as the incubation duration increased (Figure 23).

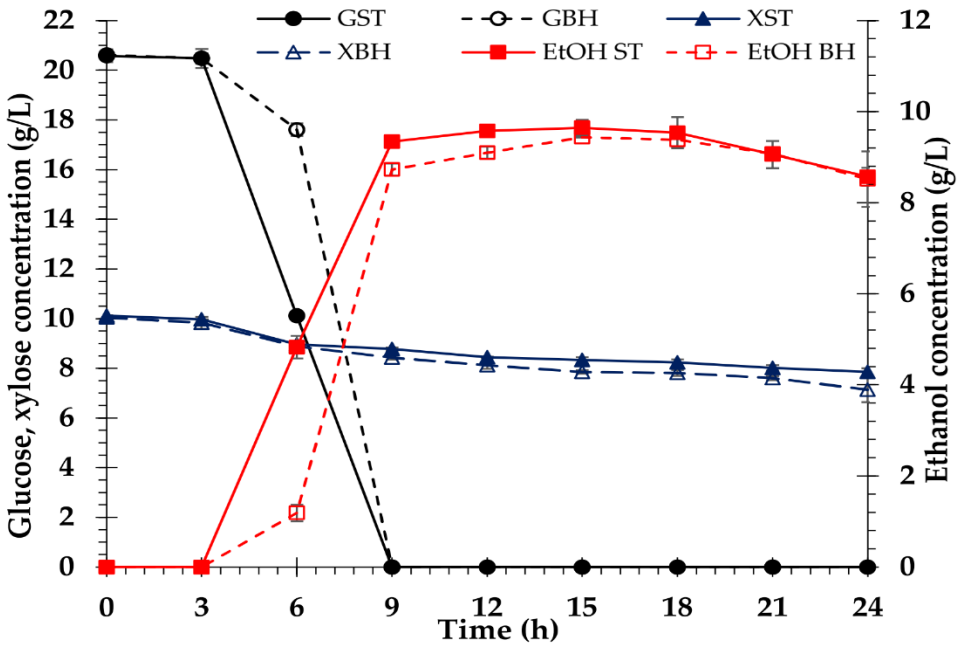

Figure 22 Profiles of sugar consumption and bioethanol production of *S. cerevisiae* TISTR 5339 in standard and biomass hydrolysate

Note: GST = glucose consumption in standard medium, GBH = glucose consumption in biomass hydrolysate medium, XST = xylose consumption in standard medium, XBH = xylose consumption in biomass hydrolysate medium, EtOH ST = ethanol in standard medium, and EtOH BH = biomass hydrolysate medium

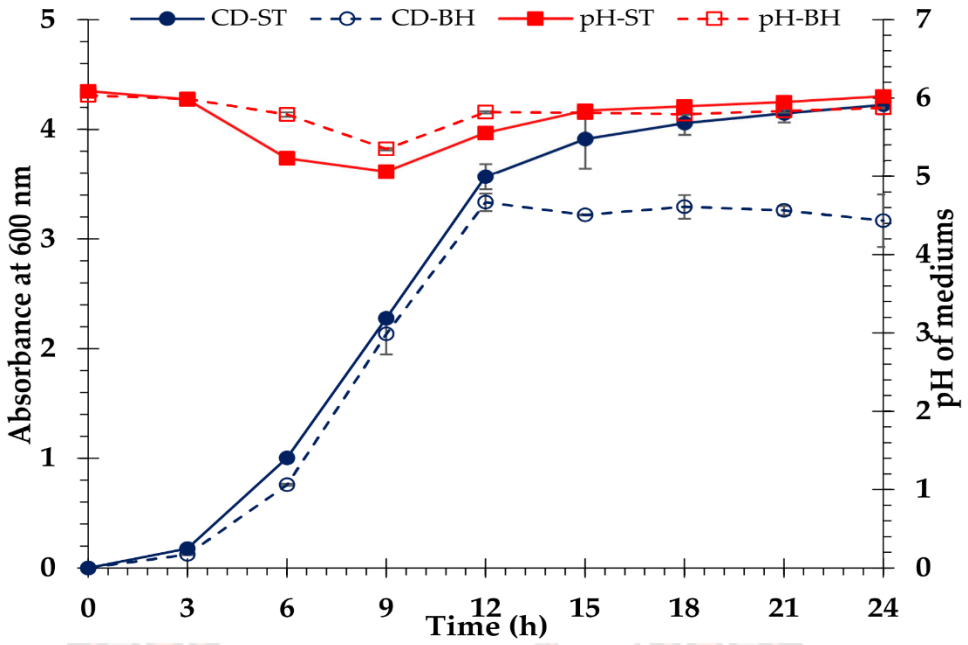

Figure 23 Cell density of *S. cerevisiae* TISTR 5339 in standard and biomass hydrolysate medium

Note: CD-ST = cell density of yeast cell in standard medium, CD-BH = cell density of yeast cell in biomass hydrolysate medium, pH-ST = the pH in standard medium, and (pH-BH) = the pH in biomass hydrolysate medium

Xylitol Production

Following ethanol production, the liquid component was precipitated using centrifugation and filtered to remove yeast cells and a variety of impurities. Subsequently, the liquid was concentrated to a xylose concentration of 20 g/L. These concentrations were then used as carbon sources in the XBH medium for xylitol production. The xylose control (XC) medium used for the evaluation was prepared using D-xylose (99% reagent grade) at a concentration of 20 g/L. *C. tropicalis* TISTR 5171 was cultured in XBH medium without detoxification and XC medium to produce xylitol. Several parameters, including xylose consumption, xylitol concentration, yeast cell proliferation, and medium pH, were examined to evaluate the xylitol producing potential of each medium. The results of these assessments are shown in Figures 24 and 25.

During the incubation period, the growth patterns of C. tropicalis TISTR 5171 and the alterations in pH observed in both XC and XBH media were comparable. After 5 days of incubation, the yeast reached its maximal growth rate in both XC and XBH media, whereas the pH varied from 5.5 to 6.1 and 5.5 to 6.6 in XC and XBH media, respectively (Figure 24).

The results demonstrate that, within 2 days of incubation, C. tropicalis TISTR 5171 entirely consumed xylose in both XC and XBH media, whereas xylitol synthesis steadily increased. Maximum xylitol yield (10.3 ± 0.1 g/L, or xylitol yield 56.6 ± 0.1%) was achieved from the XC medium after 2 days of incubation. However, the highest pro-duction of xylitol (9.6 ± 0.1 g/L, or xylitol yield 52.5 ± 0.3%) was achieved after 3 days of culture in the XBH medium. Subsequently, xylitol production steadily declined in both the XC and XBH media (Figure 25).

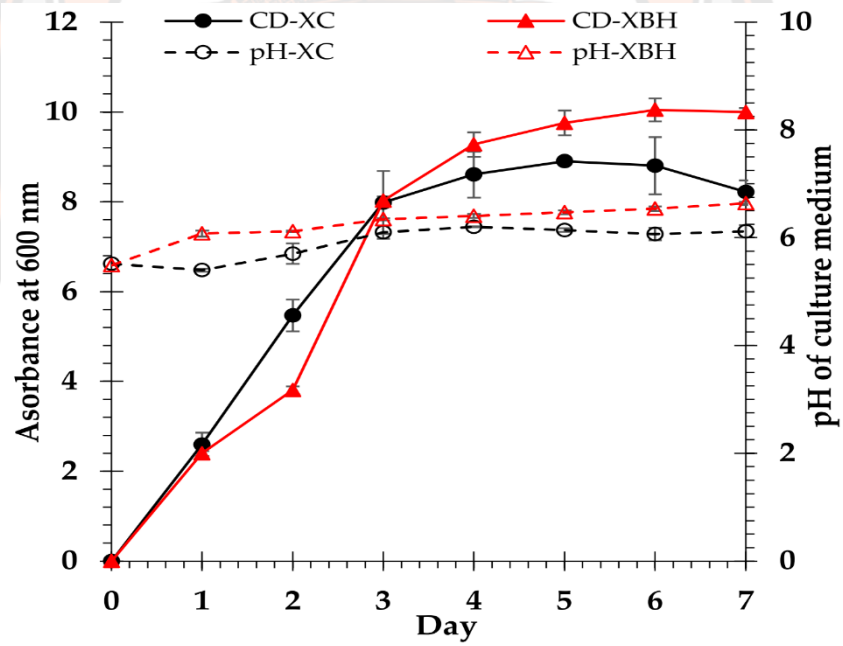

Figure 24 Cell density of *C. tropicalis* TISTR 5171 in xylose control and xylose biomass hydrolysate medium

Note: CD-XC = cell density of *C. tropicalis* TISTR 5171 in xylose control medium, CD-XBH = cell density of *C. tropicalis* TISTR 5171 in xylose biomass hydrolysate medium, pH-XC = the pH in xylose control medium, and pH-XBH = the pH in xylose biomass hydrolysate medium

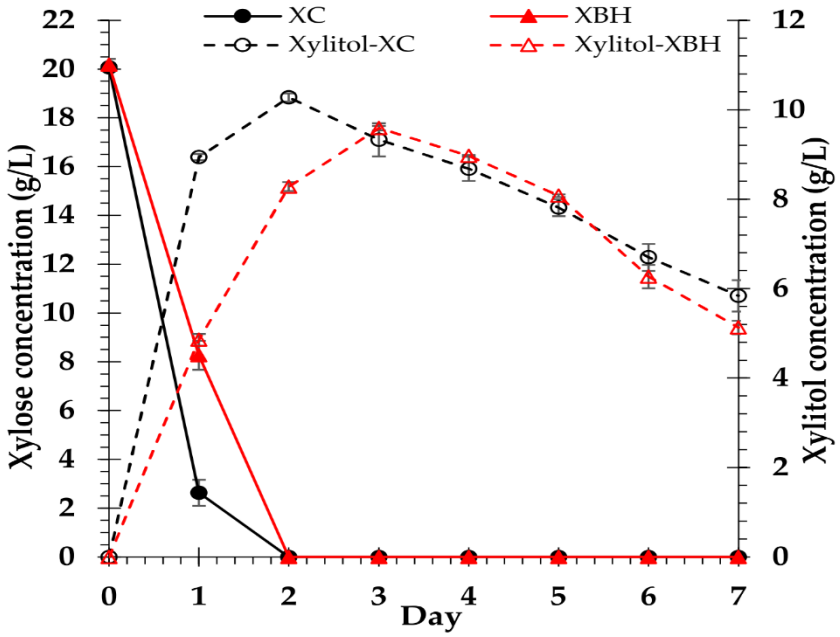

Figure 25 Profiles of xylose consumption and xylitol production of *C. tropicalis* TISTR 5171 in control and biomass hydrolysate media

Note: XC = xylose consumption in control medium, XBH = xylose consumption in biomass hydrolysate medium, Xylitol-XC = xylitol in control medium, and Xylitol-XBH = xylitol in biomass hydrolysate medium

Discussion

Effect of NaOH Concentration on Composition

Utilizing lignocellulosic material in sugar-based biorefinery platforms necess pretreatment processes to facilitate the breakdown of the recalcitrance structure of plant cell wall (Galbe & Wallberg, 2019; Wongleang et al., 2023b). Pretreatment of lignocellulosic biomass with NaOH effectively removes lignin and hemicellulose, while only partially solubilizing cellulose, depending on the reaction concentration, duration, and the environmental conditions (Modenbach & Nokes, 2014).

An increase in relative glucan content was attributable to the partial elimination of lignin and hemicellulose fractions from the sample, as observed in *Chloris barbata* (Obeng et al., 2019), two varieties of durian peel (Obeng et al., 2021), cassava stem waste (Papathoti et al., 2022), *Sida acuta* (Premjet et al., 2018b), spent coffee grounds (Wongsiridetchai et al., 2018), rice straw (Ashoor & Sukumaran, 2020), and *Sicyos angulatus* (An et al., 2021). According to our findings, the relative xylan content improved markedly at 1%-3% NaOH, whereas xylan content declined significantly ($p < 0.05$) at 4% NaOH, which may be because of the increased breakdown of hemicellulose compounds in the presence of a higher concentration of NaOH (Wongsiridetchai et al., 2018; Yan et al., 2020). At mild concentrations, NaOH (0-3%) had a minor impact on decomposing its hemicellulose during hydrothermal treatment (Sato et al., 2019), which resulted in an increase in the relative xylan content. Therefore, the increase in xylan content after NaOH pretreatment is contingent on the lignocellulosic biomass type. The phenomenon has been observed in *Sida acuta* (Premjet et al., 2018b), spent coffee grounds (Wongsiridetchai et al., 2018), rice straw (Ashoor & Sukumaran, 2020), herbaceous and woody lignocelluloses (Wang et al., 2020), Corn Cob and Sweet Sorghum Bagasse (Mafa et al., 2020), and *Sicyos angulatus* (An et al., 2021).

Delignification, which is total lignin removal (AIL and ASL) in the pretreatment process, is caused by the OH^- which liberated from NaOH, reacting and destroying internal-molecular α- and β-aryl ether and C-C bonds in lignin molecules. Consequently, increasing NaOH concentrations, which resulted in a greater amount of OH^-, had substantial impacts on lignin degradation. (Jung et al., 2020; Kim et al.,

2016b; Mohd Sukri et al., 2014; Yoo et al., 2020; Zhao et al., 2012), as demonstrated by a variety of lignocellulosic materials, including brewer's spent grain (Fernández-Delgado et al., 2019), wheat straw (Tsegaye et al., 2019b), grass waste (Yan et al., 2020), and *Pennisetum purpureum* (Haldar & Purkait, 2022). Moreover, delignification efficiencies in this study ranged from 41.9 ± 0.1% to 66.4 ± 0.5%, which is lower than the pretreatment of *V. pusilla* biomass with H_3PO_4 (54.4 ± 1.1% to 82.4 ± 1.2%) (Wongleang et al., 2023b).

However, the decrease in recovery yields of solid, xylan, and glucan can be attributed to the impact of NaOH during the weed biomass pretreatment process. This is mainly because NaOH can destroy the covalent bonds between lignin and hemicellulose within the lignin-carbohydrate complex, leading to the disintegration of both lignin and hemicellulose (Jung et al., 2020; Kim et al., 2016b; Mohd Sukri et al., 2014; Yoo et al., 2020; Zhao et al., 2012). In particular, the disintegration of H bonds in OH^- groups of carbohydrates, including glucan, xylan, mannan, arabinan, and galactan, contributed to the degradation of these sugars (Kim et al., 2016b; Yoo et al., 2020). Consequently, increasing the NaOH concentration led to greater carbohydrate degradation, which considerably reduced the recovery yields of all solids, glucan, and xylan, as observed in *Chloris barbata* (Obeng et al., 2019), grass waste (Yan et al., 2020), and durian peel (Obeng et al., 2021). Additionally, the elimination of lignin, and other chemical compounds from the feedstock had a substantial effect on the decreased amount of solid recovery at various NaOH concentrations (Haldar & Purkait, 2022; Obeng et al., 2019), which also occurred in *Sida acuta* (Premjet et al., 2018b), rice straw (Sato et al., 2019), grass waste (Yan et al., 2020), *Sicyos angulatus* (An et al., 2021), cassava stem (Papathoti et al., 2022), and *Pennisetum purpureum* (Haldar & Purkait, 2022; Manokhoon & Rangseesuriyachai, 2020).

Effect of NaOH Concentration on Hydrolysis Yield

The untreated samples generated the lowest quantity of enzymatic hydrolysis products. Lignocellulosic matter is resistant to decomposition by microorganisms and enzymes because of its rigidity and inherent recalcitrance (Kumar et al., 2020; Lorenci Woiciechowski et al., 2020; Zoghlami & Paës, 2019). After treating the feedstock with various NaOH concentrations, the yields of GR, HEG, XR, and HEX significantly

increased for each treated biomass based on the NaOH concentration. NaOH pretreatment improves the enzymatic hydrolysis yield of this biomass by breaking down the *V. pusilla* feedstock's complex structure, removing lignin and a portion of the hemicellulose that functions as a physical obstacle during hydrolysis, making carbohydrates more accessible to enzymes (Kumar et al., 2020; Mankar et al., 2021; Zhao et al., 2022; Zoghlami & Paës, 2019).

According to the results, 2% NaOH generated the highest GR and XR yields, whereas higher NaOH concentrations resulted in lower GR and XR yields. In contrast, NaOH concentrations of 3-4% produced a higher yield of HEX but had no effect on the yield of HEG. The results indicated that increasing the NaOH concentration accelerated the dissolution of lignin, hemicellulose, and cellulose, resulting in a lower solid recovery yield. A reduction in solid recovery yield had a substantial influence on sugar recovery yield (Chinwatpaiboon et al., 2023; Manokhoon & Rangseesuriyachai, 2020; Obeng et al., 2019; Obeng et al., 2021; Premjet et al., 2018b).

Effect of Temperature on Biomass Composition

The results showed that the three main compositions of the treated sample changed considerably depending on the pretreatment temperature. Decreases in AIL, ASL, and total lignin contents were also significantly ($p < 0.05$) influenced by the temperature. However, the pretreatment temperature had distinct effects on the reduction in AIL and ASL. Therefore, subjecting the feedstock to high temperature NaOH pretreatment can disrupt the complex structure of *V. pusilla* feedstocks by eliminating both lignin and hemicellulose components (Li et al., 2023a)

Compared with the raw material, the relative glucan content increased with increasing temperatures because lignin was mostly removed at higher temperatures (Obeng et al., 2019; Obeng et al., 2021). However, the hydrothermal pretreatment of biomass with NaOH (1–3%) did not have a substantial impact on hemicellulose component (Sato et al., 2019), resulting in a maximum increase in the xylan content at 110 °C and reduction at 120 °C and 130 °C because of the increased degradation of hemicellulose. In addition, as the temperature increased, the recovery yields of particulates, xylan, and glucan declined drastically, whereas lignin removal was considerably enhanced. Therefore, higher temperatures accelerated the dissolution of

solid fraction, lignin, and carbohydrates (Hoşgün & Bozan, 2020; Lee et al., 2022; Obeng et al., 2019; Obeng et al., 2021; Xu et al., 2010).

Effect of Temperature on Hydrolysis Yield

Furthermore, the yields of GR, HEG, XR, and HEX were substantially enhanced when the biomass of *V. pusilla* was treated at varying temperatures. Compared with other pretreatment temperatures, the yields of GR and XR were lowest at 130°C. However, HEG and HEX yields were highest at high temperatures, which contributed to a more severe decomposition of carbohydrates, resulting in a decline in enzymatic saccharification yields of glucose, xylose, and reducing sugar (Chinwatpaiboon et al., 2023; Obeng et al., 2019; Obeng et al., 2021). The highest amounts of GR and an average quantity of XR were obtained at 120°C. In addition, glucose had a higher recovery yield than xylose, indicating that glucose is the key product of the saccharification process and xylose is a byproduct.

The results demonstrate that both the concentration of NaOH and temperature used in the present study had a substantial effect on the alteration of all three major chemical constituents of the *V. pusilla* feedstock. Although the highest amount of HEG and HEX were obtained when the samples were pretreated with 4% NaOH at 120 °C and 2% NaOH at 130 °C, these may not be the optimal pretreatment conditions for *V. pusilla* biomass. The optimal pretreatment conditions for such biomass should generate the highest potential GR yields, which are directly proportional to the initial glucan content of the biomass. (Wongleang et al., 2023b; Yoo et al., 2017). Therefore, the optimal pretreatment of *V. pusilla* biomass in this study was 2% NaOH at 120 °C for 60 minutes, which produced the highest amount of GR. At this condition, 50.5 ± 0.1% of the lignin was removed, indicating that complete lignin elimination may not be necessary. Compared with the untreated feedstock, 2% NaOH treatment to *V. pusilla* biomass increased GR and XR yields by approximately 3.7 and 2.4 folds, respectively. Meanwhile, HEG and HEX outcomes improved by approximately 4.6 and 3.7 folds, respectively. Moreover, the GR yield of this experiment (42.4%) was slightly greater than that of a *V. pusilla* sample treated with H_3PO_4 (40.8%) (Wongleang et al., 2023b).

Effect of Pretreatment on Crystalline Structure

NaOH pretreatment not only effectively solubilized lignin and partial hemicellulose from the lignin carbohydrate complex but also altered the crystallinity, porosity, and surface area of cellulose in the treated sample (Kim et al., 2016b; Lorenci Woiciechowski et al., 2020; Mankar et al., 2021), which was also demonstrated in the present study. All treated and untreated samples maintained XRD patterns that were acceptable with cellulose I crystallinity. This implies that modifying the concentration of NaOH and temperature during the sample pretreatment did not transform cellulose I into cellulose II but increased the relative CrI of the treated materials. NaOH-aided thermal pretreatment at 75 °C to 125 °C (Zhao et al., 2022) diminishing amorphous components (glucan, xylan, arabinan, and lignin), enhancing the proportion of relative crystalline cellulose in pretreated solids, leading to a greater CrI value (Ahmed et al., 2017; Chinwatpaiboon et al., 2023; Debiagi et al., 2020; Kontogianni et al., 2019; Li et al., 2023a; Phitsuwan et al., 2016; Tsegaye et al., 2019b; Yan et al., 2020; Zhang et al., 2023).

Compared with the raw materials, the GR, HEG, XR, and HEX yields of each pretreatment condition were enhanced, which clearly indicates an increase in the relative CrI value but did not affect the enhanced enzymatic hydrolysis yield. NaOH pretreatment resulted in the removal of primarily amorphous components, coupled with considerable swelling and alteration within the crystalline sections of the cellulose in the treated samples. These modifications improved enzymatic hydrolysis yields by rendering the cellulose surface readily available to enzymes (Shahabazuddin et al., 2018; Xu et al., 2016). The effect have been observed in various alkaline pretreatments, including wheat straw (Kontogianni et al., 2019), *Chloris barbata* (Obeng et al., 2019), *Durio zibethinus* (Obeng et al., 2021), poplar (Li et al., 2023a), and sugarcane bagasse (Zhang et al., 2023).

Effect of Pretreatment on Morphological Structure

As the original lignocellulosic feedstock has a complicated and stubborn structure, it is not readily biodegradable (Mankar et al., 2021; Zhao et al., 2022). The findings suggest that changes in the surface morphology of the *V. pusilla* feedstock were significantly influenced by the NaOH concentrations and temperature employed

during the pretreatment. These modifications resulted from the NaOH pretreatment and temperature dependent breaking the bonds between the lignin-polysaccharide matrix, resulting in the solubilization of hemicellulose and lignin along with an increase in the porosity and surface area of cellulose (Mardawati et al., 2022; Obeng et al., 2019; Obeng et al., 2021; Thamsee et al., 2019; Wang et al., 2017; Wang et al., 2020; Zhang et al., 2023). Notably, adaptations enhance the efficiency of enzymatic hydrolysis by facilitating the accessibility of carbohydrates to the treated samples (Huang et al., 2017; Manokhoon & Rangseesuriyachai, 2020; Thamsee et al., 2019; Wang et al., 2021)

Ethanol Fermentation

Bioethanol can be produced from lignocellulose by fermentation of either hexose or pentose sugars, depending on the microorganism strain. The inability of *S. cerevisiae* TISTR 5339 to convert sugar from pentose to ethanol is a limiting factor in the generation of biofuels from biomass (Broda et al., 2022). In contrast with the standard medium, non-detoxified BH medium significantly affected yeast cell proliferation. Therefore, the BH medium might be contaminated with inhibitory compounds, such as phenolic compounds and aliphatic acid, which were generated during the alkaline pretreatment of the raw material preparation (Ladeira-Ázar et al., 2019; Li et al., 2023a; Łukajtis et al., 2018; Zhang et al., 2012). These inhibitors suppressed *S. cerevisiae* TISTR 5339 growths but have no impact on bioethanol production (Lee et al., 2022; Nandal et al., 2020; Premjet et al., 2018b; Toor et al., 2020; Wongleang et al., 2023b). In the present study, the fermentation medium contained both glucose and xylose. When the amount of glucose is inadequate, xylose was consumed as a carbon source, resulting in a decrease in xylose (Patiño et al., 2019; Vargas et al., 2023). Cellulosic ethanol was successfully produced utilizing *S. cerevisiae* TISTR 5339 in a non-detoxified BH medium derived from enzymatic saccharification. The maximal ethanol yield of the BH medium 89.6 ± 0.1% or 9.4 ± 0.0 g/L and the standard medium 91.8 ± 1.6% or 9.7 ± 0.2 g/L did not differ significantly.

Interestingly, the liquid hydrolysate, a byproduct of ethanol production, still contained a substantial quantity of xylose (7.1 ± 0.5 g/L), indicating the potential use of xylose as a substrate to produce other useful substances, such as xylitol.

Xylitol Production

A sustainable and cost-effective bioconversion process is essential for the successful implementation of biorefineries (Zininga et al., 2023). Therefore, xylose in the liquid hydrolysate was used as substrates for xylitol production using *C. tropicalis* TISTR 5171.

Notably, the NaOH pretreatment of lignocellulosic materials leads to delignification, resulting in the production of lignin derivatives, particularly phenolic compounds, and aliphatic acids (Ladeira-Ázar et al., 2019; Li et al., 2023a; Łukajtis et al., 2018; Zhang et al., 2012). These chemical compounds effectively inhibit yeast cell proliferation and resulted in decreased xylitol production (Bianchini et al., 2023; Vardhan et al., 2023; Zhang et al., 2012). The potential impact of inhibitory compounds may not be identifiable in our study because *C. tropicalis* TISTR 5171 grew better in the XBH medium than in the XC medium. According to the findings, both the XC and XBH media were successful in producing xylitol levels higher than 50%. Nevertheless, the xylitol yield of XC medium was slightly higher than that of XBH medium. Nonetheless, the entirety of xylose presents in both XC and XBH media was completely used. This could be attributed to the fact that the yeast cells metabolized most of the xylose in the medium, leading to an inadequate amount of xylose for xylitol production. In addition, some studies have found that xylitol output and productivity rates are constrained by low xylose concentrations (Jin et al., 2023; Misra et al., 2013; Vardhan et al., 2023).

Material Balance

Material balance calculations were conducted under the optimal NaOH-catalyzed steam pretreatment conditions to generate fermentable sugars from *V. pusilla* biomass (Figure 26). The overall process and distribution of the main components of *V. pusilla* also evaluated. The main components of the raw material were glucan, xylan, arabinan, and total lignin. After pretreatment with 2% NaOH at 120 °C for 60 minutes, the mass balance indicated approximately 57.9% recovery of the pretreated

material comprising 38.9% glucan, 12.3% xylan, and 13.8% total lignin. To evaluate the influence of pretreatment on enzymatic hydrolysis, untreated and pretreated samples were treated with cellulase, β-glucosidase, and xylanase. The results indicate a significant ($p < 0.05$) enhancement in GR, HEG, XR, and HEX yields in the pretreated biomass. After 96 hours of hydrolysis, the pretreated biomass had an HEG of 88.4% (GR = 424 g) and HEX of 74.1% (XR = 118 g). In contrast, the untreated sample displayed a HEG of 19.4% (GR = 115 g) and HEX of 20.1% (GR = 50 g). The yields of GR and XR were increased by approximately 3.7 and 2.4 times, respectively, compared with those of the raw material. Furthermore, there was an enhancement of around 4.6 and 3.7 times in the yields of HEG and HEX, respectively.

Subsequently, the BH obtained through enzymatic hydrolysis was used as a carbon source for bioethanol production by *S. cerevisiae* TISTR 5339. The results indicating a higher bioethanol yield from the pretreated biomass (194.1 g) than that from the untreated sample (52.7 g) provide evidence for the improvements through pretreatment and demonstrate the potential of non-detoxified BH as a carbon source for cellulosic ethanol production. Following the ethanol production, the liquid hydrolysate, which consisted of xylose, was used as a carbon substrate for the xylitol biosynthesis by *C. tropicalis* TISTR 5171. Notably, the xylitol output from the processed biomass (39.8 g) was roughly 2.4 times higher than the untreated sample (16.9 g).

Overall, we have demonstrated that the glucose and xylose in the BH and XBH medium, without any other detoxification processes, could be utilized as sustainable six and five-carbon sources for producing bioethanol and xylitol from *V. pusilla* feedstock, respectively. The results of this study provide vital details for the thermal pretreatment of *V. pusilla* biomass with NaOH, which is necessary for the complete utilization of six and five-carbon sugars from weed biomass to produce two value-added products (bioethanol and xylitol). To optimize the recovery of solids, enhance the hydrolysis efficiency of glucose and xylose, and improve the recovery yields of both sugars, the influence of various pretreatment parameters, including the temperature, NaOH degrees, and period, should employing a response surface methodology experimental design. However, bioethanol production can be improved by utilizing yeast strains tolerant to inhibitory or by eliminating inhibitors from the

BH before employed it in ethanol fermentation. In addition, microorganisms can become more tolerant of high ethanol concentrations through strain modifications.

To improve xylitol conversion yields, selecting microbial strains or genetically modified microorganisms that convert xylose to xylitol with greater efficacy than natural microbial strains is crucial. Additionally, the xylitol production medium needs to be optimized. The implementation of this approach is expected to optimize the efficiency of the cellulosic ethanol and xylitol production using plant-based biomass. The results highlight the potential of this simple process for producing bioethanol and xylitol, promoting the sustainable utilization of *V. pusilla* biomass, and supporting the development of a circular bioeconomy.

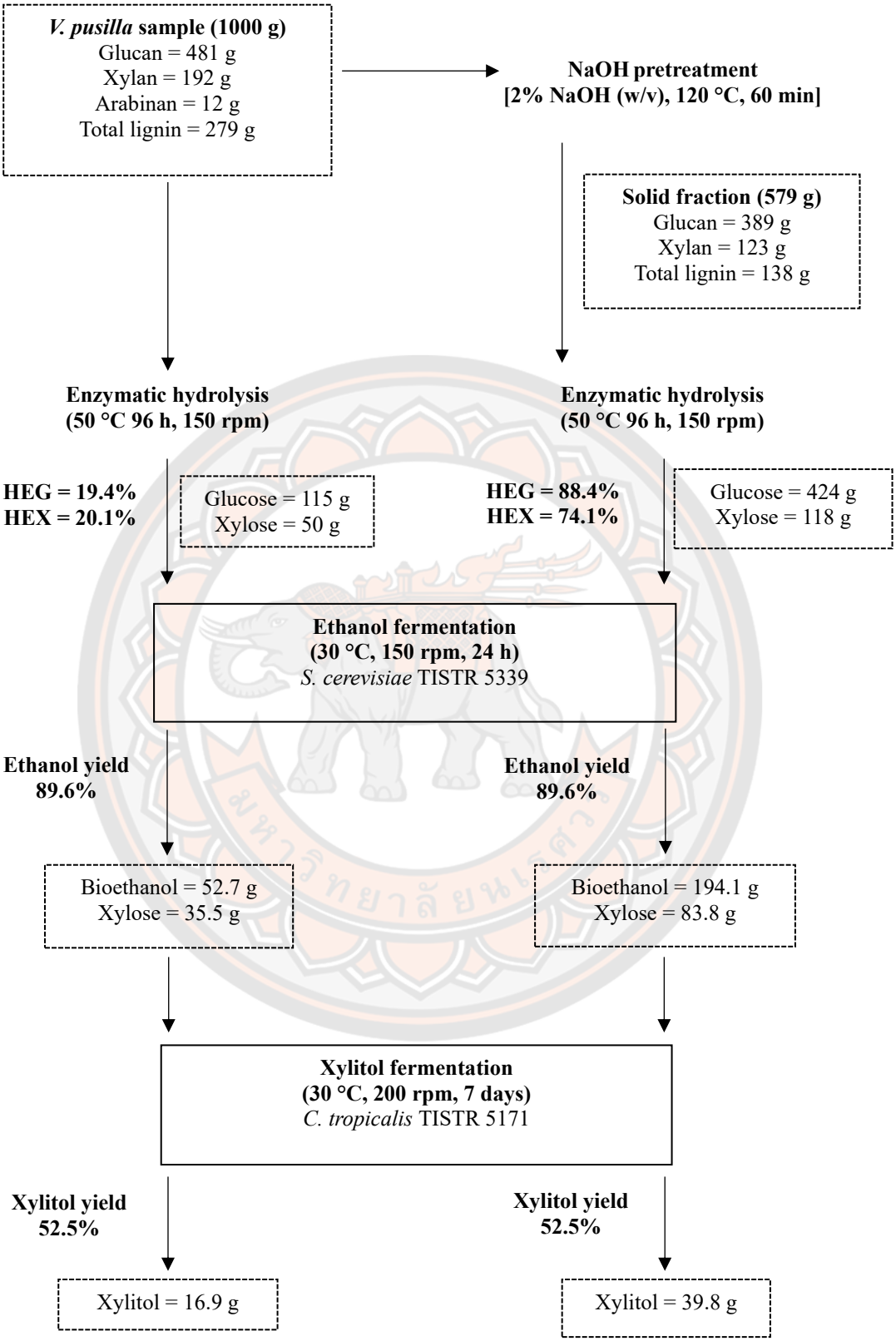

Figure 26 Material balance of *V. pusilla* biomass utilized in the fermentation of bioethanol and xylitol

Conclusions

In the current study, an autoclave was employed to perform thermal pretreatment of *V. pusilla* biomass at varying concentrations of NaOH and temperature. The synergistic action of temperatures and NaOH resulted in the breakdown of the stiff structure of *V. pusilla* biomass, resulting in delignification, an increase in cellulose porosity and surface area, and enhanced enzymatic hydrolysis yields. *V. pusilla* feedstock was efficiently pretreated with 2% NaOH at 120 °C for 60 minutes. Under these circumstances, the most excellent yields of glucose recovery (42.4 ± 0.0%) and glucose hydrolysis efficiency (88.4 ± 0.0%) were achieved. However, delignification was only 50.5 ± 0.1%, indicating that complete lignin removal is not mandatory. In addition, glucose and xylose in the BH and XBH media, respectively, could potentially be utilized as renewable hexose and pentose carbon sources to generate bioethanol and xylitol from *V. pusilla* feedstock without any additional detoxification operations. The yields of bioethanol (194.1 g) and xylitol (39.8 g) were considerably higher than those obtained from untreated biomass. Therefore, *V. pusilla* biomass has a great potential for application in a sugar platform-based biorefinery for the production of bioethanol, xylitol, or other high-value products. Nevertheless, to optimize the recovery yields of solids, glucan, xylan, hydrolysis efficiency, and the products of glucose and xylose recoveries, the pretreatment variables temperature, NaOH concentration, and duration were evaluated using statistical techniques such as RSM. Enhancing xylitol output requires improving the microbial strains used to convert xylose more efficiently into xylitol. Optimizations of the medium utilized for xylitol synthesis is also recommended.

CHAPTER V

OVERALL CONCLUSIONS

Cellulosic ethanol production is considered a promising alternative renewable feedstock for achieving sustainable development goals. Both pretreatment methods, 75% H_3PO_4 and 2% NaOH at 120 °C for 60 minutes, have shown significant improvements in sugar recovery and hydrolysis efficiency yields. Pretreatment of lignocellulosic biomass is a crucial step towards bioethanol production, leading to an increase in ethanol yield. Furthermore, a byproduct of ethanol production, still contained a substantial quantity of xylose, indicating the potential use of xylose as a substrate to produce xylitol.

Overall, these studies highlight the potential of *V. pusilla* biomass in sugar platform-based biorefineries for the production of biofuels and other valuable compounds.

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