BIOETHANOL PRODUCTION BY USING PITAYA FRUIT PEEL WASTE AS CARBON SOURCE

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By

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ABSTRACT

Leong Lai Chin

Pitaya (*Hylocereus polyrhizus*), commonly known as dragon fruit, is regarded as a promising carbon source for ethanol production because of its excellent growth rates in various soil and climate conditions. The main objective of this study was to utilize pitaya waste as a carbon source for cellulosic ethanol production. Physical pretreatment by mechanical communition of the pericarp of the pitaya, through a combination of cutting and grinding, was used. The cut material measured 1 cm, and the ground material 300 µm to 850 µm. Both cut and ground pericarp were then treated with saturated steam at 121 °C and 15 psi for 15 min. Following the hydrothermal treatment, the pericarps were subjected to enzymatic hydrolysis. Test results indicated that the cut pericarps contained the highest cellulose fraction (46.5 %) after pretreatment and consequently produced the highest level of fermentable sugars (3.86 g/L) after hydrolysis with the cellulase enzyme. From the SEM analysis, the observed perforation of the hydrothermally treated cut pericarps allowed greater enzyme adsorption and hence activity, via the increased pore volume and surface area, with the natural structure of the material better maintained than seen in the smaller particle sizes (300 µm to 850 µm). Statistical experimental designs were used to optimize the simultaneous saccharification and fermentation (SSF) process for maximum production of bioethanol from the hydrothermally treated 1 cm cut pericarps. Six critical variables; temperature, cellulase enzyme, pectinase enzyme, substrate, pH value, and inoculum size were initially selected using the Plackett-Burman design, and then suitable ranges of

these variables found by the paths of the steepest ascent. The optimized medium composition for maximum ethanol production was obtained by response surface methodology (RSM) based on a Central Composite Design (CCD). The SSF optima for pretreated pericarps under RSM were: temperature, 35.1 °C; cellulase, 0.85 mL/g; pectinase, 0.69 mL/g. Under these optimum conditions, the highest level of ethanol achieved was 7.57 g/L. The model predicted that the maximum concentration of ethanol under the optimum conditions would be 7.59 g/L, indicating that the experimental results were in close agreement with the model. Pitaya pericarp was concluded to be a suitable raw material for the production of bioethanol.

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APPROVAL SHEET

This dissertation entitled **"BIOETHANOL PRODUCTION BY USING PITAYA FRUIT PEEL WASTE AS CARBON SOURCE"** was prepared by LEONG LAI CHIN and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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It is hereby certified that **LEONG LAI CHIN** (ID No: **08UEM08132**) has completed this dissertation entitled "BIOETHANOL PRODUCTION BY USING PITAYA FRUIT PEEL WASTE AS CARBON SOURCE under the supervision of Associate Professor Dr Hii Siew Ling (Supervisor) from the Department of Chemical Engineering, Faculty of Engineering and Science, and Dr Lai Soon Onn (Co-Supervisor) from the Department of Chemical Engineering, Faculty of Engineering and Science.

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Yours truly,

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(Leong Lai Chin)

DECLARATION

I hereby declare that this dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR, or any other institution.

Name **LEONG LAI CHIN**

Date _____

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LIST OF ABBREVIATIONS

DNS	3,5- dinitrosalicylic acid
g/L	weight per volume
GC	gas chromatography
g/g	weight per weight
% w/v	percentage weight per volume
% v/v	percentage volume per volume
psi	pounds per inch
wt	weight
SSF	simultaneous saccharifiation and fermentation
SEM	scanning electron microscope
RSM	response surface methodology
CAGR	Compound Annual Growth Rate
CCD	central composite design
EU	European Union
AFEX	Ammonia fiber/freeze explosion
NDF	neutral detergent fiber
ADF	acid detergent fiber
ADL	acid detergent lignin
PGU/g	polygalacturanase unit/gram
EGU/g	endoglucanase unit/gram
ML	million litre

CHAPTER 1

INTRODUCTION

The projected decline in the non-renewable production of petroleum makes lignocellulosic-derived biomass an appealing feedstock for renewable fuel production. Suitable biomass feedstocks include agricultural deposits, wood, and food waste; typically composed of cellulose (40 % to 50 %), hemicelluloses (23 % to 35 %) and lignin (15 % to 20 %). One of the advantages of using biomass such as trees, grasses, agricultural residues, and forestry wastes, is that it can be done on a renewable basis, thus contributing to the development of a sustainable fuel industry. Another advantage is that the CO_2 emitted during the consumption of biomass-based energy sources is balanced by the CO_2 absorbed by growing biomass (Spatari et al., 2005).

Since biomass is locally available, the development of a biomass-based energy industry stimulates local job growth (Lin and Tanaka, 2006). Additionally, using biomass as an energy source repurposes otherwise unprofitable waste, such as agricultural residues, yard wastes, forestry wastes, and municipal wastes.

The USA leads the world in bioethanol production, with an annual production of 54 200 ML in 2012, followed by Brazil with an annual production of 22 900 ML the same year. There has been a substantial increase in world bioethanol production, with a Compound Annual Growth Rate (CAGR) of 15.4 % from 33 514 ML (2003) to 105 608 ML (2011) (Chin and H'ng, 2013). The US

Department of Energy offered more than USD 1 billion in funding toward lignocellulosic bioethanol projects in 2007, with the aim of reducing the biomass fuel price to USD 1.33 per gallon by 2012. In 2006, the EU provided approximately USD 68 million in support of bioethanol (Slade et al., 2009).

The production of bioethanol in Southeast Asia is forecast to reach 9 700 ML by 2015. The Southeast Asians bioethanol market generated revenues of USD 529.7 million in 2008, and aims to achieve USD 6.17 billion by 2015 (Chin and H'ng, 2013).

The Malaysian government aims to reduce net greenhouse gas emissions by producing biofuel from agricultural and forestry residues. The natural choice of feedstock being palm oil milling waste. According to the Federal Land Development Authority (FELDA), Malaysia is the second largest producer of palm oil in the world, with a production of eighteen million tons, some 37.5 % of the total world production output of forty-eight million tons. Approximately seventy million tons of biomass is produced annually in Malaysia, and 85.5 % of that production is contributed solely by the palm oil industry. The National Biofuel Policy in Malaysia aims to stimulate research and commercialize biofuels as an alternative energy source to lessen the reliance on petroleum. This initiative is supported by large local companies such as Lestari Pasifik, which has proposed to capitalize two billion ringgit in bio-refinery plants in Malaysia and Indonesia over the next five years.

Pitaya, or dragon fruit, is the common name for a large variety of warm-climate fruit of different species and genera, including the genus *Hylocereus*. Local demand for pitaya has increased in recent years and led to higher rates of production. The dragon fruit growing areas are in the states of Johor, Perak, Negeri Sembilan, Pahang, Pulau Pinang and Sabah. Due to the abundance of pitaya pericarp feedstock, the lignocellulosic composition was investigated to determine its potential for producing fermentable sugars suitable for biofuel utilization under different pretreatment conditions. The chemical composition of pitaya pericarp may vary slightly with pitaya species, soil, and climate conditions.

1.1 Problem Statement

The economical production of bioethanol is greatly inhibited by the recalcitrance of lignocelluloses to enzymatic conversion to fermentable sugars (Zhu et al., 2008). Extensive research has been conducted to increase the yield of lignocellulosic materials to bioethanol, and pretreatment to render the cellulose more accessible to enzymatic hydrolysis has been one approach. Pretreatment increases porosity, and hence the accessibility of lignocelluloses to enzyme attack; while the removal of lignin aids increases the efficiency of the enzyme (Cara et al., 2008). Inefficient pretreatment will hinder hydrolysis by the cellulase enzyme, and in extreme cases allow toxic compounds to form which inhibit the microbial metabolism (Kodali and Pogaku, 2006). Research to increase yield has focused on the optimization of the hydrolysis process and the improvement of enzyme activity (Sun and Cheng, 2002). The rate of enzymatic hydrolysis relies

on several structural features such as the crystallinity of the cellulose, surface area of the cellulose fibres, swelling of the cellulose matrix, degree of polymerization, and the formation of lignin (Detroy and Julian, 1982). It has been demonstrated that slow conversion rates and low yield are caused by low substrate concentration, and although this can be counteracted by high enzyme dosage, it is at the expense of increased production costs (Hamelinck et al., 2005).

Fermentable sugars obtained from hydrolysis can be converted to ethanol via fermentation with yeast; specifically, Saccharomyces Cerevisiae. Two possible processes may generally be applied, one involves separate hydrolysis and fermentation (SHF) reactions, and the other a simultaneous saccharification and fermentation (SSF) reaction (Cao et al., 2004). A major disadvantage of SHF is the inhibition of cellulose conversion by glucose and cellobiose, the end-products of saccharification (Wyman, 1996). SSF overcomes this by fermenting the glucose into ethanol immediately, and can increase the ethanol yield by 40% with respect to SHF. Additional benefits of SSF include higher cellulase hydrolysis rates, lower enzyme loading, and increased yeast activity if the ethanol can be continuously recovered (Picard et al., 2007). An efficient SSF reaction depends on the compatibility of reagents, enzymes, yeast concentration, temperature, etc. Hydrolysis conditions that encourage fermentation and minimize inhibitors is the aim.

1.2 Scope of Study

Pitaya fruit peel was investigated to determine the most suitable pretreatment technique to maximise cellulose digestibility. The pretreatment techniques utilized were both mechanical and hydrothermal. The dried pitaya pericarps was comminuted to attain various particle sizes and ranges of 1 cm, 850 µm to 1 mm, 600 µm to 850 µm, and 300 µm to 600 µm. The different sizes were analysed for enzymatic hydrolysis efficiency, and the morphological changes in cell wall structure caused by pretreatment observed.

Optimization of the bioethanol production process was carried out after pretreatment. A screening test involving the Plackett-Burman design was conducted on all six critical variables to identify the main factors affecting optimization targets. Operating parameters that affect fermentation, including the pH of the medium, incubation temperature, agitation speed, amount of substrate, cellulase enzyme loading, pectinase enzyme loading, and yeast extract were investigated. Response Surface Methodology (RSM), based on Central Composite Design (CCD), was applied after the determination of the significant parameters in enhancing bioethanol production from pretreated pitaya peel as the sole carbon source.

1.3 Research Objective

Pitaya peel is often considered a waste product after fruit processing. The literature is limited on dragon fruit properties, processed products and

constituents, and potential utilizations. Therefore, the objectives of this research were:

1. To evaluate pitaya waste as a possible carbon source for ethanol production after the development of a suitable pretreatment process.

2. To screen for and select significant variables affecting bioethanol production from pitaya waste.

3. To optimise the bioconversion of pretreated pitaya peels to bioethanol by the simultaneous saccharification and fermentation (SSF) process.

CHAPTER 2

LITERATURE REVIEW

2.1 Hylocereus Polyrhizus (Pitaya)

Pitaya is an introduced crop that is well established in Malaysia, Australia, Vietnam, China, Israel, Taiwan and Nicaragua. The *Hylocereus* species originated from the tropical countries of Central and South America (Barthlott and Hunt, 1993; Mizrahi et al., 1997). Table 2.1 shows the nomenclature of the pitaya fruit. The fruit has bright reddish skin with green leafy protuberances shaped like scales, and the flesh is either white or red and contains small black seeds. The flesh is juicy and delicious, but as the plant is a climbing vine cactus species, it was originally used for ornamental purposes. The flower is beautiful and has been named "Noble Woman" or "Queen of the Night". The French introduced the crop to Vietnam approximately one hundred years ago, and it was grown for the King, though it later became popular among the wealthy families of the entire country. In Vietnam, the fruit has become a major export that fetches a higher price than Durian, the "King of Fruits" in Southeast Asia.

Pitaya is a very economical crop, as it yields fruit in its second year, and reaches full production within five years. As the plant requires less nitrogen compared to most other crops, it can be grown organically using locally available organic manures and composts. For these reasons pitaya is considered a fruit crop of the

future (Gunasena and Pushpakumara, 2006; Gunasena et al., 2006). Figure 2.1 shows the use of trellises for supporting pitaya in a plantation. Pitaya cultivation is a popular industry in Malaysia, as the combination of high yield and consistent fruiting brings early and reliable income to planters, and although initial investment is comparatively high, profits are substantial for large scale plantations.

Pitaya peels are a waste product of the juicing process, and are normally discarded. Discarded peels may then become an environmental problem, particularly water pollution. In addition to being a feedstock for animals, the peels can be used in the production of bioethanol, and thereby increase the profitability of juicing. Figure 2.2 shows the pitaya fruit with pulp and peel.

TABLE 2.1 THE NOMENCLATURE OF PITAYA

Kingdom	Plantae (plants)
Sub kingdom	Tracheobionta (vascular plants)
Super division	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Magnoliopsida (dicotyledons)
Order	Caryophyllales
Family	Cactaceae (cactus family)
Subfamily	Cactoideae
Tribe	Hylocereae
Genus	*Hylocereus*
Species	*Hylocereus polyrhizus*

Sources: Britton and Rose (1963); ISB (2002); NPDC (2000)

FIGURE 2.1 PITAYA TREE WITH TRELLISES

FIGURE 2.2 HYLOCEREUS POLYRHIZUS (PITAYA) - RED SKIN AND RED PULP

2.2 Agricultural Waste as an Alternative Biomass Feedstock for Ethanol Production

Renewable fuels are being sought as an alternative to reduce the world's dependence on non-renewable resources, and thus lignocellulosic-derived biomass is regarded as a very promising feedstock for the future. Lignocellulosic biomass for the production of bioethanol is considered a financially viable replacement to food crops, and bioethanol technology is migrating from the laboratory into commercial plants. However, the conversion of food crops, comprised mostly of sugar, into bioethanol is easier compared to lignocellulosic biomass, or starchy material, because saccharification is not needed (Cardona and Sanchez, 2007). Theoretically, 1000 kg of raw sugar will yield 500 L of ethanol (Balat, 2011). Table 2.2 summarizes the production yields of ethanol, and the sugar to ethanol conversion efficiency as a percentage for lignocellulosic biomass, and feedstocks containing starch and sugar.

TABLE 2.2 COMPARISON OF SUGAR AND ETHANOL CONTENT FOR THE THREE MAIN TYPES OF BIOETHANOL FEEDSTOCKS

Feedstock Category	Feedstock	Cellulose content (%)	Ethanol yield (L/L)		Conversion efficiency (%)	Citation
			Actual ethanol yield	Theoretical ethanol yield		
Sugar containing substrate	Sugar cane juice	12	70	78	90	Baucum et al., 2006; Coelho, 2005
	Sugar beet	18	100	116	86	Berg, 2001
Starchy substrate	Cassava	32	178	207	86	Wang, 2002
	Sweet sorghum	15	80	97	82	Rao et al., 2004
	Wheat	66	350	427	82	Rao et al., 2004
	Corn	70	403	452	89	Baker and Zahniser, 2006
Lignocellulosic biomass	Cane bagasse	33	140	213	66	Moreira, 2000
	Wheat straw	36	140	233	60	Ballesteros et al., 2006

Feedstock Category	Feedstock	Cellulose content (%)	Ethanol yield (L/L)		Conversion efficiency (%)	Citation
			Actual ethanol yield	Theoretical ethanol yield		
	Corn stalk	35	130	226	63	Demirbas, 2005
	Switchgrass	39	201	252	80	Bakker et al., 2004
	Populusnigra	35	151	226	64	Ballesteros et al., 2004
	Eucalyptus globulus	36	138	232	59	Ballesteros et al., 2004
	Brassica carinata	33	128	213	60	Ballesteros et al., 2004

Bioethanol is usually produced from sugar cane, beet, or starchy cereals. However, the low cost and accessibility of lignocellulosic material has prompted recent research into its use as a feedstock for bioethanol production. Lignocellulosic biomass has the advantages of low alternate utility, low cost, and shared land opportunity cost. Many researchers are engaged in transforming lignocellulosic biomass, such as leaves, stalks and other plant wastes into ethanol.

However, converting lignocellulosic biomass into monomeric sugar is more difficult than converting from starch. In table 2.2, switch grass had the highest ethanol yield of 201 L/L. Lignocellulose materials such as *Populousniagra* and *Eucalyptus Globule*, containing mostly woody substance, recorded conversion efficiencies of 59 % to 64 % (Ballesteros et al., 2004). Conversion efficiency is higher in sugary and starchy materials than in lignocellulosic materials.

Biomass feedstocks include agricultural residues, wood and solid waste. Biomass is typically composed of cellulose (40 % to 50 %), hemicelluloses (23 % to 35 %) and lignin (15 % to 20 %). One of the advantages of using the biomass of trees, grasses, agricultural residues, and forestry wastes, is that they are produced on a renewable basis, and contribute to the development of a sustainable fuel industry. Another advantage is that the CO_2 emitted during the consumption of biomass-based energy source can be balanced by the CO_2 absorbed from the atmosphere during biomass growth (Spatari et al., 2005). Biomass is produced locally, and hence the development of a biomass based energy industry will stimulate the local job market (Lin and Tanaka, 2006).

Converting biomass waste to energy is a profitable way of solving waste problems. Additionally, Lignocellulosic biomass is abundant and require less agricultural inputs for its production.

2.3 Ethanol as a Biofuel

Biofuels are fuels formed through the physical, chemical, and biological treatment of biomass. They differ by way of chemical species, source biomass, and manufacturing methods (Kevin, Lishan and Mark, 2006). Biomass derived ethanol is one of the best alternative fuels for the transportation sector, and is now widely used in the U.S. as a partial gasoline replacement to reduce petroleum usage and tailpipe emissions. In 2008, the U.S. ethanol industry generated nine billion gallons of ethanol, a volume 5.4 times greater than the past decade (RFA, 2009). In the United States, ethanol production is currently dominated by corn grain feedstocks. United States policies enforce the quantitative use of biofuels derived from cellulosic biomass, with the Energy Independence and Security Act (EISA) of 2007 increasing quotas within the Renewable Fuels Standard (RFS). The RFS demanded that 100 million gallons of cellulosic biofuel be used in 2010, and projects the usage of sixteen billion gallons by 2022 (EISA, 2007). By 2022, twenty-one of the thirty-six billion gallons of renewable fuel under the RFS is required to be from advanced biofuels, i.e. not produced from corn starch. Cellulosic ethanol is expected to provide a large fraction of the required advanced biofuel because it is nearer to commercialization than many other advanced biofuels, such as those produced through the thermochemical platform and chemical catalysis (Brodeur-Campbell et al., 2008).

Around Asia, countries such as China are producing ethanol using corn stover. The production of cellulosic ethanol in China is operated by SunOpta Inc., owned by the China Resources Alcohol Corporation. Japanese manufacturers Nippon Oil Corporation and Toyota Motor Corporation have expanded their research on biofuels derived from lignocellulose materials, and this confederation proposes to produce fifty million gallons of bioethanol per year by 2014 (Chin and H'ng, 2013).

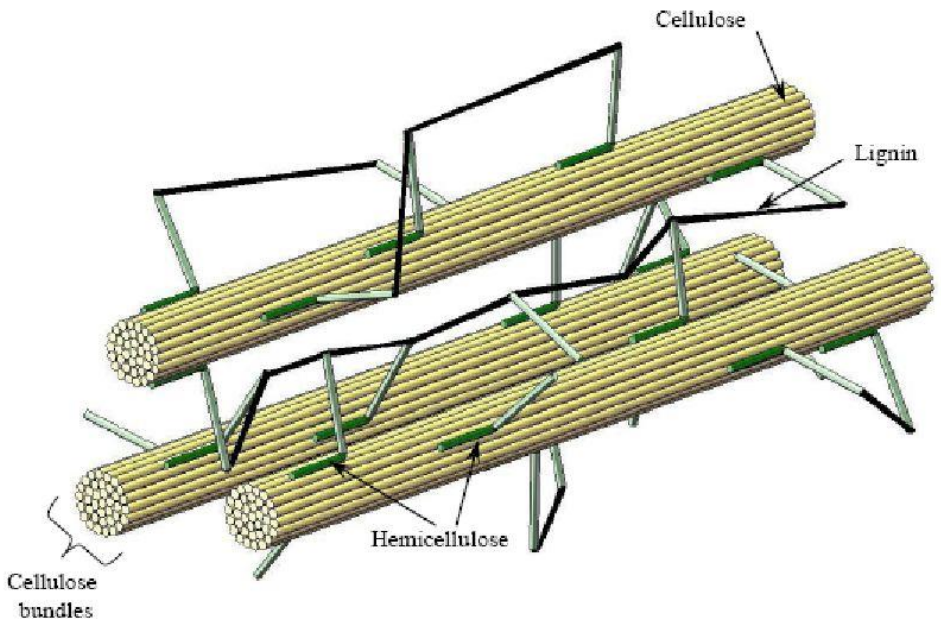
The Malaysian government has envisioned biofuel as one of the main energy sources of the country, and this is reflected by the National Biofuel Policy. The Five-Fuel Diversification Policy reifies renewable energy as the fifth most utilized fuel, behind natural gas, coal, oil, and hydropower. The National Biofuel Policy was developed in 2006 to boost the production of biofuels for local use, and to export biodiesel from palm oil. In 2007, the Government postponed the biodiesel project indefinitely due to financial issues.

In 2011, the National Biomass Strategy 2020 was launched to promote biofuel, with the ultimate aim of stimulating Malaysia's gross national income (GNI) through biomass commercial activities and creating sustainable jobs. This strategy plans to produce bioethanol from lignocellulosic biomass like oil palm, and then extend to other waste sources. By 2020, bioethanol production is estimated to increase to 100 million tonnes, contributed to primarily by waste from plantation land. Oil palm biomass is targeted to contribute an additional twenty million tonnes. Malaysia is seen to have great potential as a bioethanol producer, and the bioethanol market can cause significant impact on the nation's economy, while reducing greenhouse emissions.

Before 2020, all cars in Malaysia must utilize 10% bioethanol in commercial fuel to reduce the emission of gasses that cause the greenhouse effect, by decree of the National Biomass Strategy 2020. It is estimated that one million tonnes of bioethanol is required per year for local use to accommodate this Strategy. The first biomass plant is expected to commence operation between 2013 and 2015 (AIM, 2011).

2.4 Composition of Lignocellulosic Materials

Lignocellulosic biomass is a highly heterogeneous, and its composition varies with source. Cellulose, hemicellulose, and lignin made up the three major components in lignocellulosic materials, Figure 2.3 (Shaw, 1999). Table 2.3 shows the comparison of lignocelluloses from several dry sources.



(Source: Shaw, 1999)

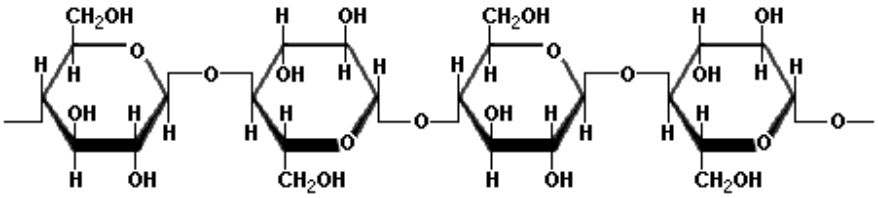
FIGURE 2.3 ARRANGEMENT OF CELLULOSE MICROFIBRILS IN PLANT CELL WALLS

TABLE 2.3 LIGNOCELLULOSE COMPOSITION

Materials	Percentage dry weight (%)			
	Cellulose	Hemicellulose	Lignin	Others (ash, fatty acids, protein, etc.)
Corn fibre	15	35	8	42
Corn cob	45	35	15	5
Corn stover	0	25	17	18
Rice straw	35	25	12	28
Wheat straw	30	50	20	-
Cane baggasse	40	24	25	11
Switch grass	45	30	12	13

(Source: Saha, 2003)

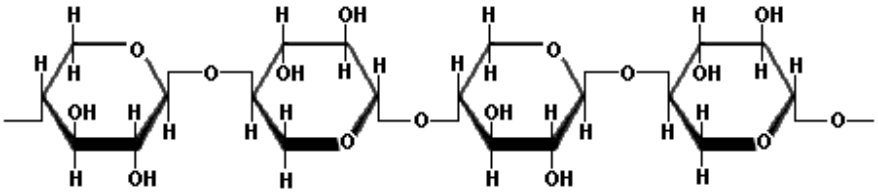
Typically, 30 % to 50 % by weight is made up of cellulose (Figure 2.4) which is a linear, crystalline polymer of glucose molecules, linked through β-(1→4)-glycoside bonds.



(Source: Gibbons et al., 2002)

FIGURE 2.4 STRUCTURE OF CELLULOSE

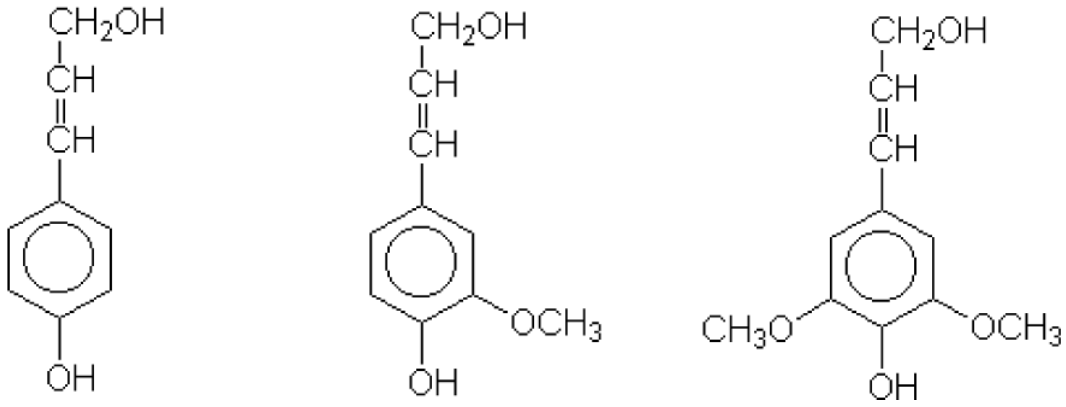
A further 20 % to 35 % is made up of hemicelluloses. Hemicellulose (Figure 2.5) is a heteropolymer made up of hexoses (glucose, galactose, and mannose), pentoses (xylose and arabinose), sugar acids (glucuronic acid), and other organic acids (acetic, ferulic acids, etc.). The majority of hemicelluloses are xylans, polysaccharides with backbone chains of xylose units (Wiselogel, 1996). Agricultural feedstocks derived from grass crops – corn stover, bagasse, switchgrass, etc – predominately contain xylans. Hemicelluloses can also be glucan, arabinan, mannan, and galactan based, like those commonly found in wood biomass. Hardwood hemicelluloses (oak, maple, etc.) are richer in xylans, while softwoods have more glucomannans (Saha, 2003).



(Source: Gibbons et al., 2002)

FIGURE 2.5 STRUCTURE OF HEMICELLULOSE

A large fraction of the remainder (2 % to 30 %) is made up of lignin (Figure 2.6), an insoluble phenylpropene polymer attached to the hemicellulose.



(Left to Right: p-coumaryl alcohol, coniferyl alcohol, and sinaply alcohol.)
(Source: Nagele et al., 2005)

FIGURE 2.6 CHEMICAL STRUCTURE OF LIGNIN MONOMERS

Finally, small fractions of ash, protein, soluble phenolic compounds and fatty acids make up the remainder (Wyman 1996). The main substrates for the production of ethanol is the hemicellulose and cellulose fractions because they are substantial sources of potentially fermentable sugars.

2.5 Conversion of Lignocellulosic Biomass to Ethanol

The conversion of lignocellulosic biomass to ethanol comprises four stages as described in Table 2.4 (Mosier et al., 2005).

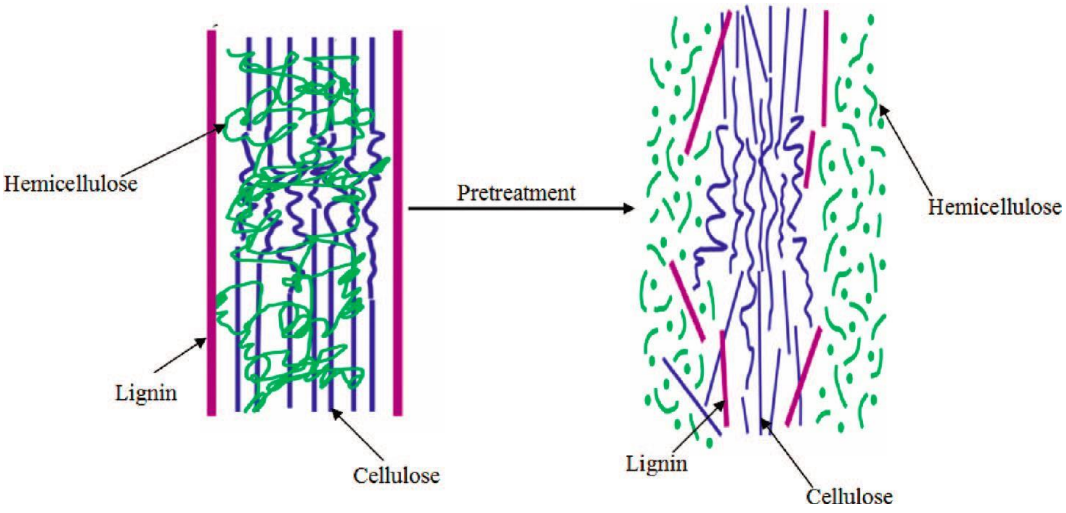
TABLE 2.4 STAGES INVOLVED IN BIOETHANOL PRODUCTION FROM BIOMASS FEEDSTOCK

Stage	Purpose
Pretreatment	Separation of lignin from cellulose and hemicelluloses fractions and "open-up" the structure for easier hydrolysis.
Hydrolysis/Saccharification	Breakdown of the polysaccharide fractions into fermentable monosaccharides.
Fermentation	Conversion of fermentable sugars into ethanol by biocatalyst (*S. cerevisiae, Z. mobilis* etc.)
Product separation/Purification	Separation and concentration of ethanol from fermentation mix.

Cellulosic ethanol production has yet to be commercialized as each step poses different challenges. However, several companies, including Iogen and Abengoa Bioenergy, have developed pilot plants for ethanol production (Williams, 2005).

2.6 Pretreatment

Pretreatment is a crucial step that breaks the extensive connections between cellulose, hemicellulose and lignin components, making them more accessible to enzymatic attack. Pretreatment technologies, including comminution, ammonia fibre explosion pyrolysis, steam explosion, acid pretreatment, and alkaline pretreatment have been intensively researched to increase the enzymatic digestibility of lignocellulose (Piskorz et al., 1989; Chang et al., 1997; Negro et al., 2003; Alizadeh et al., 2005; Sun and Cheng, 2005). Pretreatment processes can be physical, chemical or a hybrid of both, with the goal to alter or remove structural and compositional factors that hinder hydrolysis of cell wall polysaccharides into fermentable sugars, Figure 2.7. The structure of cellulose, a glucose polymer, favours the tight packing of polymer chains. The resultant structures are crystalline, water insoluble, and highly resistant to depolymerisation. Hemicellulose bonds with cellulose micro-fibrils, forming a network that reduces the penetration of depolymerising agents. The presence of lignin further impedes hydrolysis (Mosier et al., 2005). Harsher conditions are thus required to expose the two polysaccharides to hydrolysis. Pretreatment is the primary technical and economic bottleneck in large scale manufacture of cellulosic ethanol (Wyman, 2007). An energy and cost efficient pretreatment method needs to be developed for commercially viable ethanol production. Summarized below are the pretreatment techniques that have been explored.



(Source: Mosier et al., 2005)

FIGURE 2.7 SCHEMATIC SHOWING THE EFFECT OF PRETREATMENT ON THE PLANT CELL WALL

2.6.1 PHYSICAL PRETREATMENTS

Mechanical pretreatments reduce feedstock particle size and increase the surface area available for enzymatic hydrolysis (Mosier et al., 2005); examples include ball milling and compression milling. The primary issue associated with physical pretreatments is the relatively high energy cost.

Heating can be used to accelerate the reaction rate, and microwave irradiation is an effective mechanism. An electromagnetic field applied directly to the medium allows uniform and selective heating, and assures reproducibility of reactions (Kappe, 2005). Other cited advantages include the ability to start and stop the process instantaneously and a reduction in the energy requirements of the process (Gabriel et al.,1998; Datta, 2001).

2.6.2 CHEMICAL PRETREATMENTS

Acid pretreatment

Cellulose and hemicellulose release their constituent sugars under acid hydrolysis; hence, dilute acids can be used to partially break down the polymers. Sulfuric acid hydrolysis is used for hemicellulose degradation in the commercial manufacture of furfural (Zeitsch, 2000). The use of other mineral acids (nitric, hydrochloric and phosphoric acid) has been explored (Mosier et al., 2005). Unfortunately, there are several downsides to acid pretreatment. Firstly, strong acids corrode containment vessels, necessitating the use of special acid-resistant materials, and significantly adding to initial and replacement costs. Secondly, the medium must be neutralized before proceeding to fermentation. Lastly, the acids used tend to degrade sugars upon release, and convert them to fermentation inhibitors such as furfural and hydroxymethylfurfural.

Alkaline pretreatment

The major mechanism of alkaline pretreatment is the saponification of the intermolecular ester bonds that crosslink hemicellulose and other components. Alkaline pretreatment results in a disruption of the lignin structure, an increase in internal surface area, and a decrease in cellulose crystallinity (Sun and Cheng, 2002). Aqueous ammonia depolymerizes lignin and cleaves lignin-carbohydrate linkages. It also changes the phase of the crystal structure of the cellulose, thus improving digestibility. Ammonia pretreatment is a percolation

process wherein 5 % to 15 % ammonia is contacted with the biomass in a column reactor at 160 °C to 180 °C (Mosier et al., 2005). High levels of delignification were reported upon AFEX treatment of hardwood (Yoon, 1995) and agricultural residues (Iyer, 1996), as well as substantial improvements in enzyme digestibility (Kim, 2003).

2.6.3 HYDROTHERMAL PRETREATMENTS

Hydrothermal pretreatment uses hot water at elevated pressure (greater than the saturation pressure) to ensure that the water remains in liquid phase. Water acts as a solvent and a reactant at the same time. Water as a reactant is an environmentally friendly alternative to corrosive acids and toxic solvents (Bobyleter, 1994). The ionization of water decreases with temperature until a critical point, while the dielectric constant of water decreases monotonically with increasing temperature (Sierra et al., 2008). This behaviour of water with temperature favours the hydrolysis of hemicelluloses and leads to the cleavage of ether and ester bonds. Hydrothermal treatment of lignocellulosic biomass generates acid from the acetyl groups of the hemicelluloses, which catalyses the hydrolysis and solubilisation of hemicelluloses. Recovery of xylose from biomass can be as high as 88 % to 98 %. The structural alterations due to the removal of the hemicelluloses increases the accessibility and enzymatic hydrolysis of cellulose. This process liberates organic acids from the biomass and the pH of the reaction medium decreases during treatment.

The pH should be maintained between pH 4 and pH 7 during the pretreatment to avoid the formation of inhibitors. This minimises the build-up of

monosaccharides and degradation products that catalyse hydrolysis of the cellulosic material during pretreatment (Kohlmann et al., 1995; Weil et al., 1997; Mosier et al., 2005a; Hendriks et al., 2008; Laxman et al., 2008).

Two common hydrothermal pretreatments are uncatalysed steam explosion and liquid hot water pretreatment. Uncatalysed steam explosion uses pressurised steam which has been found to hydrolyse hemicellulose. The process has been commercially applied to the manufacture of fibre-board and other products (De Long, 1981; Ballesteros et al., 2006).

Steam explosion pretreatment requires heating of the biomass in the presence of steam-saturated water (about 200 °C and 1.5 MPa), leading to organic acid generation. This acid hydrolyses some of the hemicelluloses and alters the lignin structure. The structural changes in the biomass after the treatment enhance its enzymatic digestibility (Sierra et al., 2008). If rapid pressure change is applied after auto-hydrolysis, the water and liquid content in the biomass will explosively vaporise, shattering the biomass in a popcorn-like effect and consequently increasing surface area. This approach combines both chemical and mechanical pretreatment into one step. Low energy input and negligible environmental effects are the major advantages of the process. Steam explosion sometimes will not break down lignin completely and feedstock must usually be in particle sizes. Steam explosion also produces some inhibitory compounds that interfere with subsequent enzymatic hydrolysis and fermentation steps (Laxman et al., 2008; Sierra et al., 2008). The addition of sulfur dioxide or carbon dioxide during steam explosion treatment can improve the enzymatic hydrolysis of biomass by making the

pretreatment environment more acidic. SO_2 forms sulfuric acid and CO_2 forms carbonic acid. The limitation of these methods is a lower yield of hemicellulose sugars (Overend and Chornet, 1987; Duff and Murray 1996).

Hot water pretreatment is carried out by contacting biomass with water heated to beyond its boiling point (Kohlman, 1995). The process can use co-current, counter-current, or cross flow through reactors (Mosier et al., 2005). The water-biomass contact is maintained for up to fifteen minutes at temperatures of 200 °C to 230 °C. Hydrolysis of the resultant liquid is carried out to recover sugars. Biomass source and lignin content have been found to be key factors in recovery yields (Mosier et al., 2005). Hot water-based pretreatment has been a strong focus at the Laboratory of Renewable Resources Engineering at Purdue, where the development of a controlled-pH, liquid hot water pretreatment process was subsequently patented (Ladisch, 1998).

2.7 Saccharification

Following pretreatment, lignocellulosic polysaccharides are more vulnerable to chemical or enzymatic hydrolysis. The goal of saccharification is to further break down lignocellulose into constituent sugars – glucose, xylose, arabinose, etc. – at high concentrations for fermentation. The hydrolysis is carried out using acids or cellulose degrading enzymes (cellulases) (Lin, 2006). Key technical challenges at this step include sugar degradation, the high cost of cellulase enzyme (Wyman, 2007), and the formation of undesirable by-products such as furfural in the case of acid hydrolysis (Wyman, 1999).

2.8 Fermentation of Bioethanol

Following the pretreatment and saccharification process, the released fermentable sugars become a potential substrate for subsequent fermentation processes. Two widely used fermentation techniques for bioethanol production are described below.

Separate enzymatic hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation (SHF) refers to the process whereby the enzymatic hydrolysis (saccharification) of polysaccharides and microbial fermentation are performed sequentially and orthogonally as shown in Figure 2.8. The major advantage of this method is that each process can be carried out in its own optimum conditions (Saha et al., 2005; Olsson et al., 2006). However, one drawback of the SHF process is the inhibition of cellulose activity by the released sugars, cellobiose and glucose. Cellulase activity is reduced by 60 % at cellobiose concentrations as low as 6 g/L. Glucose decreases cellulase activity as well, but less strongly. However, glucose is a strong inhibitor for β-glucosidase, with activity reductions of 75 % at glucose concentrations of 3 g/L (Philippidis et al., 1993; Philippidis and Smith, 1995).

The probability of contamination is high, as separate vessels are used for hydrolysis and fermentation. The hydrolysis process is long and the released sugars provide a suitable environment for contamination by naturally-occurring microbes. Enzyme preparation is another source of potential contamination, as it is difficult to sterilize enzymes on a large scale.

Sterilisation by filtering is expensive and autoclaving is unsuitable because the heat also denatures the enzymes (Taherzadeh and Karimi, 2007). Antibiotics cannot be added to the hydrolysis reactor because they may affect the growth and fermentation of microorganisms in the subsequent fermentation steps.

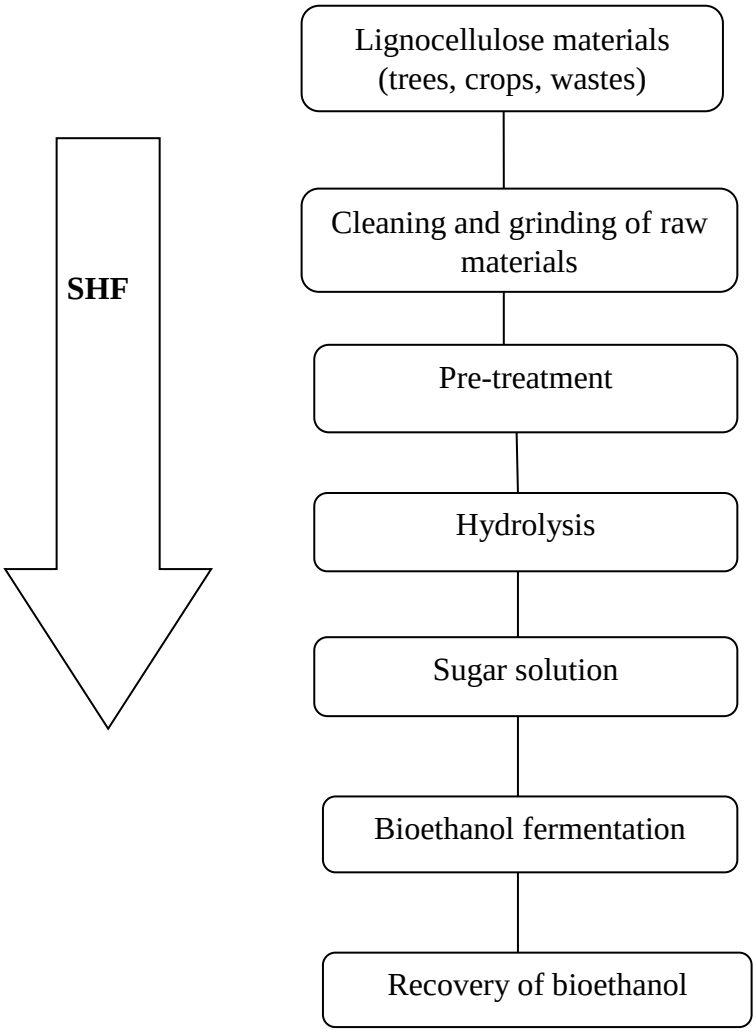


FIGURE 2.8 SCHEMATIC DEMONSTRATION OF SEPARATE SACCHARIFICATION AND FERMENTATION (SHF) PROCESS

Simultaneous Saccharification and Fermentation (SSF)

The SSF process combines the steps of hydrolysis and fermentation by introducing the biocatalyst into the same medium as where saccharification

takes place. Figure 2.9 demonstrates the flow of the SSF process. A key advantage of this process is that it helps overcome the inhibition of the cellulase enzyme by reducing the concentration of the products glucose and cellobiose, through fermentation. Rapid utilization of glucose as it is formed allows higher fermentation rates, yields, and final ethanol titers. The presence of ethanol also helps eliminate contaminating microbes (Wyman, 1999). One drawback of SSF is that enzymatic hydrolysis and fermentation have to be performed under compromised conditions, particularly with respect to pH and temperature. The optimum temperature and pH for enzyme activity and fermentation are always different. The optimal temperature for enzyme activity is typically higher than that of fermentation. Hydrolysis is usually the rate-limiting component in SSF (Philippidis and Smith, 1995).

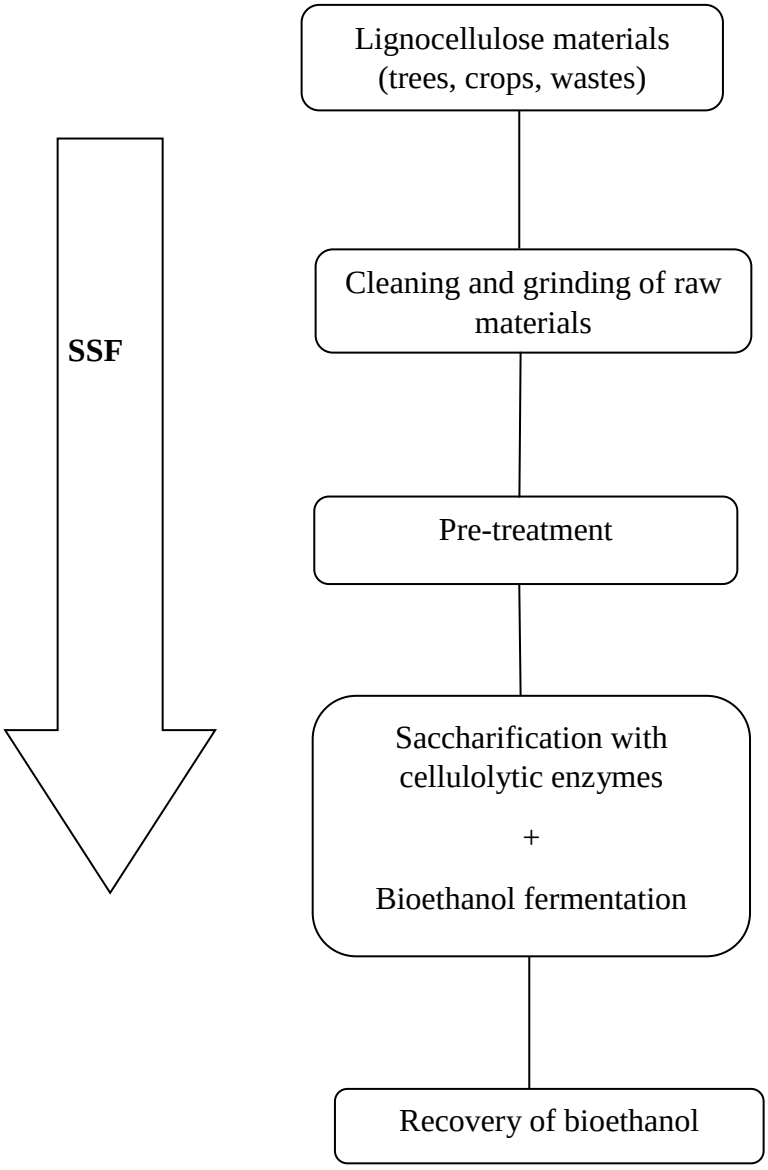


FIGURE 2.9 SCHEMATIC DEMONSTRATION OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) PROCESS

2.9 Saccharomyces Cerevisiae as Ethanol-fermenting Organism

S. cerevisiae (Baker's yeast) has gained attention as a potential biocatalyst for cellulosic ethanol fermentation due to its comparatively high yields and tolerance to higher alcohol concentrations in contrast to bacteria and other yeasts. *Saccharomyces cerevisiae* can naturally ferment hexose monosaccharides (glucose, mannose) and disaccharides (sucrose, maltose) into

ethanol, producing concentrations as high as 18% of the fermentation broth and displaying less sensitivity to inhibitors. After the breakdown of the sugars into pyruvate through glycolysis, *S. cerevisiae* converts pyruvate into acetaldehyde through the action of the enzyme pyruvate decarboxylase, and releasing a molecule of CO2 in the process. Acetaldehyde is subsequently reduced by alcohol dehydrogenase to form ethanol (Varga et al., 2004; Lin and Tanaka, 2006).

2.10 Concluding Remarks

Biomass is an attractive feedstock for fuel production and has been the world's fourth largest energy source after coal, oil and natural gas. Although the conversion of lignocellulosic material to ethanol shows promise for the sustainable production of biofuel, more research is needed to produce commercially viable and environmentally friendly methods.

Ethanol production from lignocellulosic material involves three stages; pretreatment of the lignocellulosic material, the saccharification of the lignocellulosic material to sugars, and the production of ethanol by sugar fermentation. In recent years, a lot of valuable work has been done to optimize this process and yielded tremendous improvements. Ideally, a pretreatment method should meet the following requirements: have low cost, reduce or remove the maximum amount of extractives and inhibitors including lignin, retain the majority of the polysaccharides, consume little energy, consume few chemicals, and produce no pollution. The saccharification and fermentation

steps also have optimisations that must be considered, and more research is needed to improve the performance of each step to maximise ethanol output.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Materials

The commercial enzymes pectinase (Pectinex Ultra SP) and cellulase (Celluclast 1.5 L) were purchased from Science Technics (M) Sdn. Bhd, Malaysia, and soy peptone, dextrose, and yeast extract from Difco Laboratories Oxoid (Malaysia). Ethylene glycol monoethyl ester, cetyltrimethyl ammonium bromide, hydroxide pellets are from R&M Chemicals (United Kingdom), while sodium borate decahydrate, sodium sulphide, disodium hydrogen phosphate, and potassium sodium tartrate are from Systerm (Malaysia). Phenol (detached crystals), hydrochloric acid, and sulphuric acid are from Merck (Malaysia), and 3, 5-dinitrosalicylic acid was sourced from Sigma (USA).

3.2 Pretreatments of Samples

Fresh dragon fruit pericarp samples (less than 3 months old) were obtained from a wet market in Petaling Jaya, Malaysia. The samples were cleaned of debris and washed, and then soaked and washed until the pH tested neutral. Samples were frozen (-20 ºC) until used.

Samples of pericarp were thawed and dried overnight at 60 ºC in an oven until the weight remained constant. The fully dried pericarps were then comminuted to a variety of particle sizes. Dried substrate was communited by a

combination of chopping and grinding, and then sieved to attain different particle sizes and ranges; 1 cm, 850 µm to 1 mm, 600 µm to 850 um, and 300 µm to 600 µm. Another batch of mechanically pretreated samples then subjected to hydrothermal pretreatment using an autoclave at 121 ºC and 15 psi for 15 min. Sterility was ensured by using the autoclave as a general pretreatment approach.

3.3 Lignocellulosic Substrate

The chemical composition of the mechanically pretreated and combination pretreated *H. polyrhizus* samples were analysed for hemicellulose, cellulose, and lignin content. The Van Soest method was applied for the determination of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) (Van Soest et al., 1991). NDF content provides an estimation of the hemicellulose, cellulose, and lignin contents on sample. ADF evaluates cellulose and lignin components. Table 3.1 and Table 3.2 show the preparation formula for NDF and ADF respectively. Concentrated sulphuric acid was used in the ADL determination.

TABLE 3.1 PREPARATION OF NDF SOLUTION

Chemical Reagents	Amount
Sodium laurel sulphate	30 g
Disodium dihydrogenethylenediaminetetracetate	18.61 g
Sodium borate decahydrate	6.81 g
Disodium hydrogen phospate	4.56 g
Etoxy ethanol	10 Ml

*all chemicals above were mixed in 1 L water and the solution adjusted to pH 7.0.

TABLE 3.2 PREPARATION OF ADF SOLUTION

Chemical reagents	Amount
Cetyltrimethylammonium bromide	20 g
0.5 M sulphuric acid	1.0 L

3.4 Enzymatic Hydrolysis

A volume of 1.5 L of Celluclast was prepared from commercial *Trichodermareesei* cellulose, containing *endo*-glucanases, *exo*-glucanases, cellobiohydrolases, and *β*-glucosidases. Cellulase enzyme concentrate is usually used for cellulose hydrolysis. This product is a brownish liquid with a density of approximately 1.22 g/mL, and declared activity of 700 EGU/g. Optimum conditions of activity are between pH 4.5 to pH 6 and 50 °C to

60 °C. Pectinex is formed from a mixture of the enzymes pectintranseliminase, polygalacturonase, and pectinesterase. Pectinex has a declared activity of 43 PGU/g. These enzymes also exhibit optimum activity around pH 4.5 and 50 °C. The total reaction volume for each experiment was 100 mL. Cellulase and pectinex enzymes (1 mL/100mL) were added and then mixed with the substrate (1 g/L). The reaction vessels (flasks) were covered with cotton wrapped in cheesecloth and incubated in a rotary shaker at 200 rpm and 45 ºC for 28 h. Samples were withdrawn every 4 h and fermentable sugar content analysed (Ballesteros et al., 2004).

3.5 Simulataneous Saccharification and Fermentation Process (SSF)

The SSF process was used as it was identified as the most effective for attaining the highest concentration of fermentable sugars after hydrolysis. Dried pretreated pericarps were supplemented with yeast extract (10 g/L) and peptone (20 g/L) in a 250 mL conical flask and then autoclaved for 15 min at 121 °C. Pectinex and cellulase were then added to the flasks. The flasks were inoculated with yeast *(Saccharomyces cerevisiae*) and stirred for 18 h at 37 °C and 150 rpm. Flasks were sealed using cotton wrapped with cloth to establish micro-aerobic conditions. During the fermentation process 5 mL of the culture broth was drawn periodically for analysis. Culture samples were centrifuged at 9000 rpm for 5 min. The supernatant liquid was used in the subsequent pH, residual fermentable sugar concentration, and ethanol concentration analysis (Ballesteros et al., 2000).

3.6 Analytical Methods

The fermentable sugars released were determined using the 3, 5-dinitrosalicyclic acid method (DNS acid method) of Miller. During fermentation, the supernatant was again used for analysis of fermentable sugars and ethanol concentration.

All experiments were performed in triplicate and the results presented in the form of mean ± standard deviation. The statistical software Statistical Analysis System (SAS) was used to evaluate the significance of the experimental results.

3.6.1 DETERMINATION OF SUGAR YIELD

The soluble fermentable sugars were measured using Miller's method. The DNS acid solution was prepared using: DNS acid, 10 g/L; phenol, 2 g/L; sodium sulphide, 0.5 g/L; sodium hydroxide, 10 g/L; and sodium potassium tartarate, 182 g/L.

Two drops of 1 N NaOH, and 1 mL of DNS solution were added to 1 mL of diluted sample in a test tube. This mixture was then placed in a 100 °C water bath and boiled for exactly 5 min. The mixture was then immediately cooled under running tap water. 10 mL of distilled water was then added into the solution. The colour developed fully after 20 min, and the absorbance was read to be 540 nm. A standard curve for glucose determination was tabulated to measure the concentration of fermentable sugar (Figure 3.1). Standard curve plotting has high accuracy and satisfactory determination of coefficient (R^2)

values (R^2= 0.9912) (Akin-Osanaiye et al., 2005; Firoz et al., 2012 and Teerapatr et al., 2013).

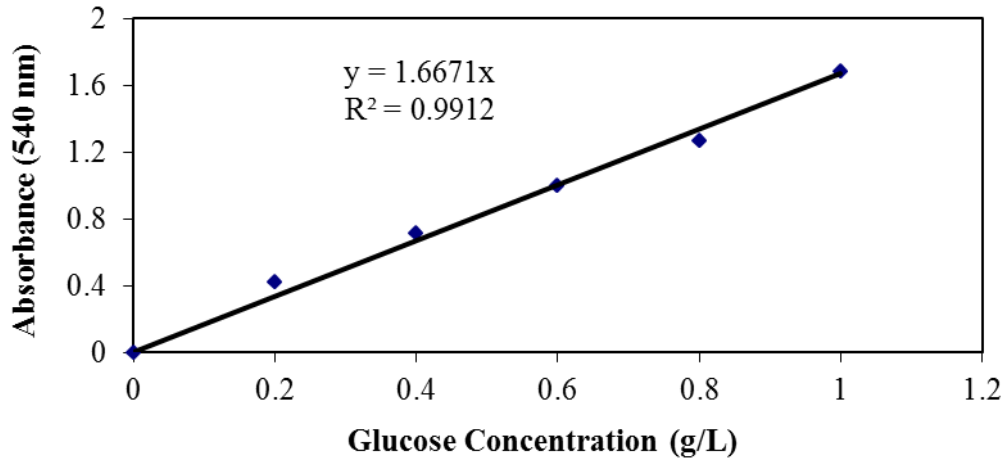


FIGURE 3.1 STANDARD CURVE OF GLUCOSE

3.6.2 ETHANOL DETERMINATION

The soluble ethanol concentration of the 3 mL sample was analysed using gas chromatography (GC) in a capillary column (Zebron ZB-Wax *plus*) under a flame ionization detector. Samples were analysed for ethanol concentration using peak-area based calibration curves. Samples from the fermentation broth were centrifuged and then filtered using an Acrodisc® 25 mm syringe filter with 0.20 µL Supor® membrane (USA). Filtered aliquots were then injected into the GC apparatus at below operating conditions as shown in Table 3.3 (Chang et al., 1997).

The ethanol standard curve was constructed (Figure 3.2) from the GC results.

TABLE 3.3 OPERATING CONDITIONS OF GC

Type of detector	**Flame Ionisation Detector**
Oven temperature	200 °C
Detector temperature	280 °C
N_2 flow rate	30 mL min^{-1}
H_2 flow rate	40 mL min^{-1}
Air flow rate	400 mL min^{-1}
Run time	11.5 min

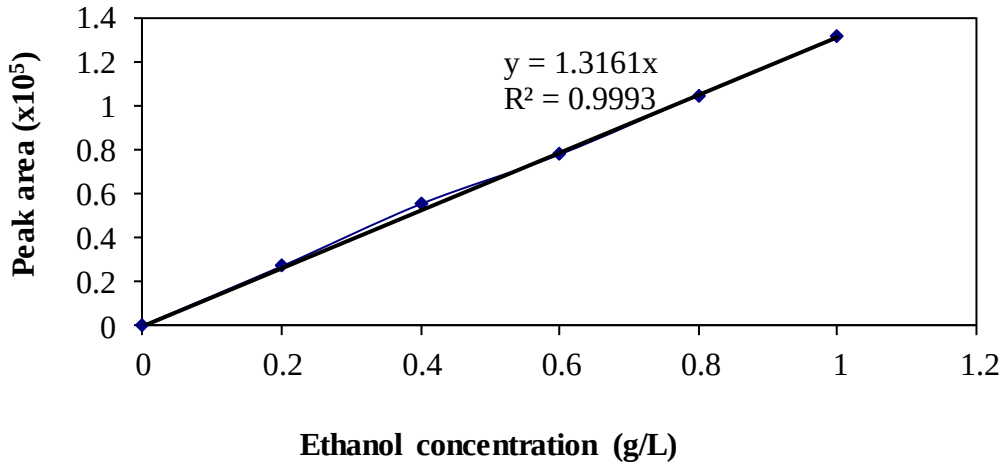


FIGURE 3.2 STANDARD CURVE FOR ETHANOL DETERMINATION

3.6.3 ETHANOL PRODUCTIVITY DETERMINATION

Ethanol yield and ethanol productivity per gram of pretreated pericarp were calculated using Equations 3.1 and 3.2.

$$Ethanol\ yield\ (g\ ethanol/g\ pericarp) = \frac{Maximum\ ethanol\ concentration\ \ (g/\mathrm{L})}{Initial\ amount\ of\ \ dry\ pretreated\ pericarp\ (\mathrm{g/L})}$$

EQUATION 3.1

$$Ethanol\ productivity\ (g\ ethanol/g\ pericarp/day) = \frac{Maximum\ ethanol\ yield\ (g/g)}{Time\ taken\ to\ achieve\ maximum\ yield\ (day)}$$

EQUATION 3.2 (LJUNGGREN, 2005)

3.7 Observation by Scanning Electron Microscope (SEM)

A Hitachi S-3400N Scanning Electron Microscopy was used to observe the morphological changes of the cell walls of pretreated samples. Pretreated pericarp was fixed onto a circular specimen holder with a double-sided carbon tape and then viewed with an accelerating voltage of 20 kV.

3.8 Statistical Analysis

All experiments were carried out in triplicate and results were analysed using SAS ® Proprietary Software Release 6.12. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used for comparison among pretreatment methods. Error bars were calculated using Microsoft Excel.

Statistical experimental designs were used to develop the simultaneous saccharification and fermentation process by *Sacchromyces Cerevisiae*. The critical nutrients were initially selected using a Plackett-Burman design and then quantities refined by using the path of steepest ascent method. The optimised medium composition for maximum ethanol production was

obtained by response surface methodology (RSM) based on a Central Composite Design (CCD) (Maache-Rezzoug et al., 2011).

CHAPTER 4

ENZYMATIC HYDROLYSIS OF H. POLYRHIZUS PERICARPS FOR PRODUCTION OF FERMENTABLE SUGAR

4.1 Introduction

The biomass of lignocellulose materials contains organic polymers such as cellulose, hemicellulose and lignin. Cellulose is found mainly in the secondary cell wall of plants and is the major structural component of higher plants. Cellulose is water insoluble and impermeable due to its crystalline structure, and the highly associated microfibrils act as an outside matrix to protect the inner environment of the plant cell (Goyal et al., 1991). This crystalline structure is a major inhibitor of cell wall hydrolysis (Aisan et al., 1997). Hemicelluloses are a heterogeneous group of polysaccharides and include four basic types: D-xyloglucans, D-xylans, D-mannans, and D-galactans. The heterogeneous monomers and linkages of hemicellulose spatially hinder enzyme attachment, reducing the effectiveness of the hemicellulase enzyme during hydrolysis (Robyt, 1997). Lignin is predominately located in the middle lamella of the plant cell wall, cross-linked with hemicellulose. Lignin hinders enzymatic hydrolysis of the carbohydrate polymer (Pordesimo et al., 2005).

Therefore, pretreatment steps are crucial to rendering lignocellulose materials more accessible to enzymatic hydrolysis. Pretreatment steps can increase porosity and remove lignin, subsequently making the lignocelluloses more

accessible to enzyme attack (Cara et al., 2008). Pretreatment results in more fermentable sugars and aids the feasibility of bioethanol production.

Dried pitaya pericarps were subjected to mechanical communition and hydrothermal pretreatment prior to enzymatic hydrolysis and fermentation, with the purpose of producing more fermentable sugars.

4.2 Materials and Methods

4.2.1 PRETREATMENT OF DRIED PEELS

Physical Pretreatment

Physical pretreatments were conducted at room temperature. Dried substrate was communited by a combination of chopping and grinding, and the resulting material sieved to attain the particle sizes and ranges of 1 cm, 850 µm to 1 mm, 600 µm to 850 µm and 300 µm to 600 µm.

Hydrothermal Pretreatment

Each particle size of pericarp was packed into individual beakers and sealed with aluminium foil. The samples were autoclaved using high-pressure steam (15 psi) at 121 ºC for 15 min. The hydrothermal pretreated pericarps were then dried in an oven at 60 ºC until weight remained constant.

4.2.2 INOCULUM PREPARATION

High analytical grade *Saccharomyces cerevisiae* was purchased from Merck (Malaysia). For the inoculum preparation of *S. cerevisiae*, 1 g of yeast powder was added to 100 mL YPD medium consisting of: yeast extract, 10 g/L; dextrose, 20 g/L; peptone, 20 g/L. The flasks were incubated in a rotary shaker at 37 ºC and 200 rpm for 16 h.

4.2.3 ENZYMATIC HYDROLYSIS PROCESS

Enzymatic hydrolysis was carried out under the following conditions: 1 g of pretreated pitaya pericarp in a working volume of 100 mL with a volume fraction of 1 % of the filter-sterilised enzymes cellulase (Celluclast ® 1.5 L from Novozymes, Denmark) and pectinase (Pectinex ® ULTRA SP-L from Novozymes, Denmark), was agitated at 200 rpm and 45 ºC. Samples were withdrawn periodically over 28 h. The sample supernatants were analysed for fermentable sugar concentrations. All processes were conducted in triplicate.

4.2.4 DETERMINATION OF FERMENTABLE SUGAR CONCENTRATIONS

The soluble fermentable sugars were measured using the DNS acid method. The DNS solution was prepared using: dinitrosalicylic acid, 10 g/L; phenol, 2 g/L; sodium sulphide, 0.5 g/L; sodium hydroxide, 10 g/L; and sodium potassium tartarate, 182 g/L. A volume of 1 mL of DNS reagent and two drops of 1 N NaOH were added to 1 mL of diluted sample in a test tube, and this

mixture was placed in a water bath and boiled for exactly 5 min. The mixture was then immediately cooled in ice water. A volume of 10 mL of distilled water was then added. After 20 min of colour development, the absorbance of the suspension was measured at 540 nm.

The determination of fermentable sugars was carried out according to the methodology described in Section 3.6.1. The statistical significance of the effect of pretreatments on enzymatic hydrolysis yield was determined by ANOVA and Duncan multiple range tests.

4.2.5 FIBRE DETERMINATION

The fibre content of dry pericarps was determined by the analysis of Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL). During the NDF process, the washed off fraction contained soluble cell contents like carbohydrates, lipids, pectin, starch, soluble proteins, and non-protein nitrogen. The remaining fraction contained hemicellulose, proteins bound to cell walls, cellulose, lignin, and recalcitrant materials. ADF solution was used to dissolve hemicellulose, cell solubles and soluble minerals, and leave behind a residue of cellulose and lignin. During ADL, cellulose was washed off, leaving only lignin and recalcitrant materials. Triplications were done on all fibre fractions. The NDF, ADF, and ADL tests was carried out according to Van Soest et al. (1991). The components determined by the NDF, ADF, and ADL tests are summarized below:

$$NDF = Cellulose + Hemicellulose + Lignin + Mineral\ Ash$$

EQUATION 4.1

$$ADF = Cellulose + Lignin + Mineral\ Ash$$

EQUATION 4.2

$$ADL = Lignin + Mineral\ Ash$$

EQUATION 4.3

$$Cellulose\ content = ADF - ADL$$

EQUATION 4.4

$$Hemicellulose\ content = NDF - ADF$$

EQUATION 4.5

Determination of Neutral Detergent Fibre (NDF)

Dried pitaya pericarps of weight 1.0 g were mixed with 100 mL NDF solution, 2 mL of decalin, and 0.5 g of sodium sulphate. The mixture was then boiled for 1 h, then filtered through a sintered filter funnel, rinsed with hot water, and then acetone. The residues were collected and dried in an oven at 105 °C overnight. The next day, the residues were left in a desiccator for few hours until constant weight was achieved. NDF was calculated by Equation 4.6, where X_1 is the original weight of pericarps in grams, and X_2 is the weight of residue after filtration in grams.

$$NDF\ (\%) = \frac{X2}{X1} \times 100$$

EQUATION 4.6

Determination of Acid Detergent Fibre (ADF)

In the ADF test, the dried pericarps (1.0 g) were digested under reflux using 0.5 M sulphuric acid for 1 h at boiling point. The mixture was then filtered and rinsed with hot water and acetone. The residues were dried in an oven (105 °C) overnight, and weighed until weight was constant. The ADF value was tabulated based on Equation 4.7, where X_1 is the original weight of the pericarp in grams, and $X_{2'}$ is the weight of the residue in grams.

$$ADF\ (\%) = \frac{X2'}{X1} \times 100$$

EQUATION 4.7

Determination of Lignin Content

The ADL was analysed from the non-dried and non-ash residues of the ADF test. The ADFs were treated with 72 % sulphuric acid at room temperature for 4 h to dissolve the cellulose, leaving the lignin as the residue (Effland, 1977). The residues were rinsed with water and dried in an oven overnight (105 °C). Constant dry weight was achieved before putting residues in a furnace (550 °C) for 3 h. The weight loss registered after the furnace represented the lignin content. The lignin content was calculated by Equation 4.8, where $X_{2'}$ is the sample weight in grams, and X_3 is the weight of the residue after filtration in grams. Specific cell wall components (cellulose and hemicelluloses) were calculated using Equations 4 and 5.

$$Lignin\ (\%) = \frac{X3}{X2'} \times 100$$

EQUATION 4.8

4.2.6 SEM ANALYSIS

A Hitachi S-3400N Scanning Electron Microscope was used to observe the morphological changes of the cell wall as a result of pretreatment. Pretreated samples were freeze dried for imaging. Samples were sputter coated with gold prior to examination to improve image quality and to avoid charging the specimens. The freeze dried samples were mounted onto a circular specimen holders using double-sided carbon tape and then viewed with an accelerating voltage of 20 kV. The original images were stored in TIFF format. They were converted to JPEG format with brightness, contrast, and gamma corrected before electronic transmission. No other image enhancement or modifications were applied.

4.3 Results and Discussion

4.3.1 CHEMICAL COMPOSITION ANALYSIS

The chemical composition of *H. polyrhizus* differs by growth location, season, harvesting method, and analytical procedure. Table 4.1 shows the hemicellulose, cellulose and lignin content after the application of different pretreatments on varying sizes of dried pericarp.

Combined hydrothermal and mechanical pretreatment increased the total available lignocellulosic material compared to mechanical pretreatment alone. Mechanical pretreated samples contained 27.15 % hemicellulose and 42.18 % cellulose by dry weight, while the hydrothermally and mechanically pretreated samples contained 29.28 % hemicellulose and 46.47 % cellulose by dry

weight. This suggested that autoclaved 1 cm samples minimised the loss of cellulose (46.47 % cellulose), the main precursor in bioethanol production from lignocellulosic materials. Statistically, there was a large difference between the biomass composition results of the mechanical and combined pretreatment techniques ($p > 0.05$).

The grinding process does not reduce particle sizes in a uniform manner (Bridgeman et al., 2007). This is evident from the fluctuation of cellulose content with particle sizes from 850 µm to 300 µm, with or without hydrothermal pretreatments as in Table 4.1. The highest hemicellulose (29.28 %) and cellulose (46.47 %) content resulted from cutting (1 cm) and autoclaving of the biomass sample. Autoclaving represents a good compromise between enhancing the accessibility of the *H. polyrhizus* for enzyme attack and avoiding high sugar loss due to extreme temperature (Pordesimo et al., 2005), while serving as a sterilisation step. This became the pretreatment of choice, and subsequent enzymatic hydrolysis experiments were conducted using 1 cm cut and autoclaved biomass.

Relative lignin content was found to increase with increasing particle size, which has been reported in the literature as undesirable for enzymatic hydrolysis, as lignin can absorb enzymes and lead to an irreversible loss of enzyme activity (Eriksson et al., 2002). However, this result was not supported by the current study, and will be discussed. Other reports have stated that particle size reduction does not necessarily yield higher cellulose and lower lignin levels, with larger particle sizes yielding a higher proportion of lignin, cellulose, and hemicellulose (Bridgeman et al., 2007).

Smaller particle sizes contain more soluble organic molecules along with inorganic matter, while larger particle sizes retain a higher carbon and volatile substance content, which means a higher calorific value that will encourage greater fermentation yields (Bridgeman et al., 2007). Residual lignin is not universally considered to negatively impact on the enzymatic hydrolysis process (Teramoto et al., 2008). The lignin content in the pretreated biomass of *H. polyrhizus* was not considered a hindrance to enzymatic hydrolysis, and therefore not a crucial recalcitrance factor of the pretreatment process.

TABLE 4.1 CHEMICAL COMPOSITION OF H.POLYRHIZUS PERICARP

Pretreatment conditions	Hemicellulose (%)	Cellulose (%)	Ligni (%)
Mechanical Treatment			
1cm	27.15 [b]	42.18 [b]	20.31 [a]
850 µm to 1 mm	24.81 [d]	39.39 [f]	15.81 [d]
600 µm to 850 µm	21.95 [f]	40.63 [d]	11.86 [f]
300 µm to 600 µm	19.95 [h]	35.33 [h]	10.81 [h]
Hydrothermal and Mechanical Treatment			
1 cm	29.28 [a]	46.47 [a]	18.85 [b]
850 µm to 1 mm	25.21 [c]	40.38 [e]	16.39 [e]
600 µm to 850 µm	23.47 [e]	41.15 [c]	15.34 [c]
300 µm to 600 µm	20.88 [g]	37.22 [g]	10.72 [g]

Mean values marked by different letters in same column are significantly different at the $\alpha = 0.05$ level.

4.3.2 SCANNING ELECTRON MICROSCOPE (SEM) CHARACTERIZATION

SEM examinations were carried out to determine which pretreatment led to the most suitable residual to be used for enzymatic hydrolysis and the subsequent fermentation. The goal was to visually assess the 'openness' of the structure of the samples as an indicator of porosity.

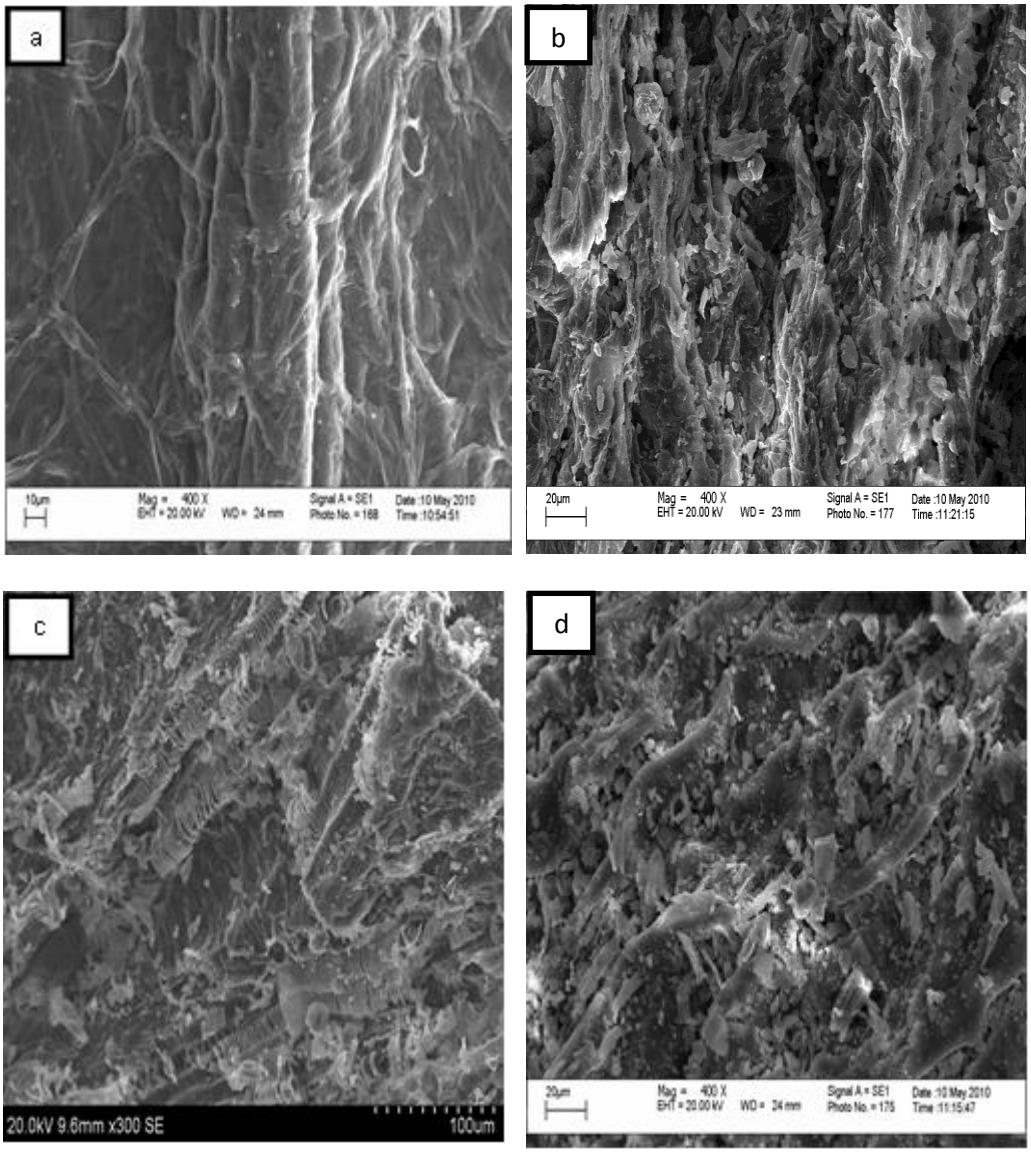
Figures 4.1 and 4.2 show the structural changes after communition, with and without hydrothermal pretreatment, respectively. SEM successfully captured the morphological and structural changes after pretreatments, and revealed the significant effect pretreatment had on the structure of the pericarp fibres. Figure 4.1 shows the morphology of 1 cm cut pericarp. The structure remained relatively rigid, with ordered fibres still glued together by fibrils. These fibrils were found to be distributed in a relatively compact zone and formed a "mat-like" morphology.

The topographies in Figures 4.1 (b, c, d) show scratches on the surface of the communited pericarps due to a distortion of crystallinity and the depolymerisation effect. Size reduction did not correlate with the increased production of fermentable sugar. This was either because communition resulted in an extensive reduction in cellulose content, or that the grinding was insufficient to stimulate sugar production (Berlin et al 2006; Bridgeman et al., 2007).

The combined communition and hydothermal pretreatment rendered the pericarps most susceptible to enzymatic attack, as it reduced the crystallinity of the cellulose and increased the proportion of amorphous phases. Figure 4.2 (a) shows the structural changes associated with autoclaving 1 cm cut pericarp, with portions of the biomass more ragged and loosened than the original samples (Figure 4.1 (a)). This structural change may explain why this pretreatment resulted in the highest sugar content, with the increased pore size and greater surface area, without the severe structural disruption of grinding, allowing greater enzyme absorption and attack.

Swelling and large numbers of perforations can be seen on the rough surfaces of the smaller autoclaved pericarps (Figures 4.2 (b, c, d)). The SEM images show wrinkle formations on the surface of the smaller autoclaved particle sizes (Figure 4.2 (b, c, d)). The swelling may be an indicator that the cellulose has been destroyed through thermal degradation and severe physical rupture, resulting in a reduced efficiency of enzymatic hydrolysis.

Lignin droplets on the surface due to lignin suspension were not seen in the SEM images of samples pretreated hydrothermally and mechanically. Structural lignin changes leading to porous spaces were not reasoned to be a main factor in the resultant efficiency of hydrothermal or mechanical pretreatment (Koo et al., 2011).



Symbols: a, 1 cm; b, 850 µm to 1 mm; c, 600 µm to 850 µm;

d, 300 µm to 600 µm.

FIGURE 4.1 SEM OF H. POLYRHIZUS PERICARPS PRETREATED BY MECHANICAL COMMUNITION

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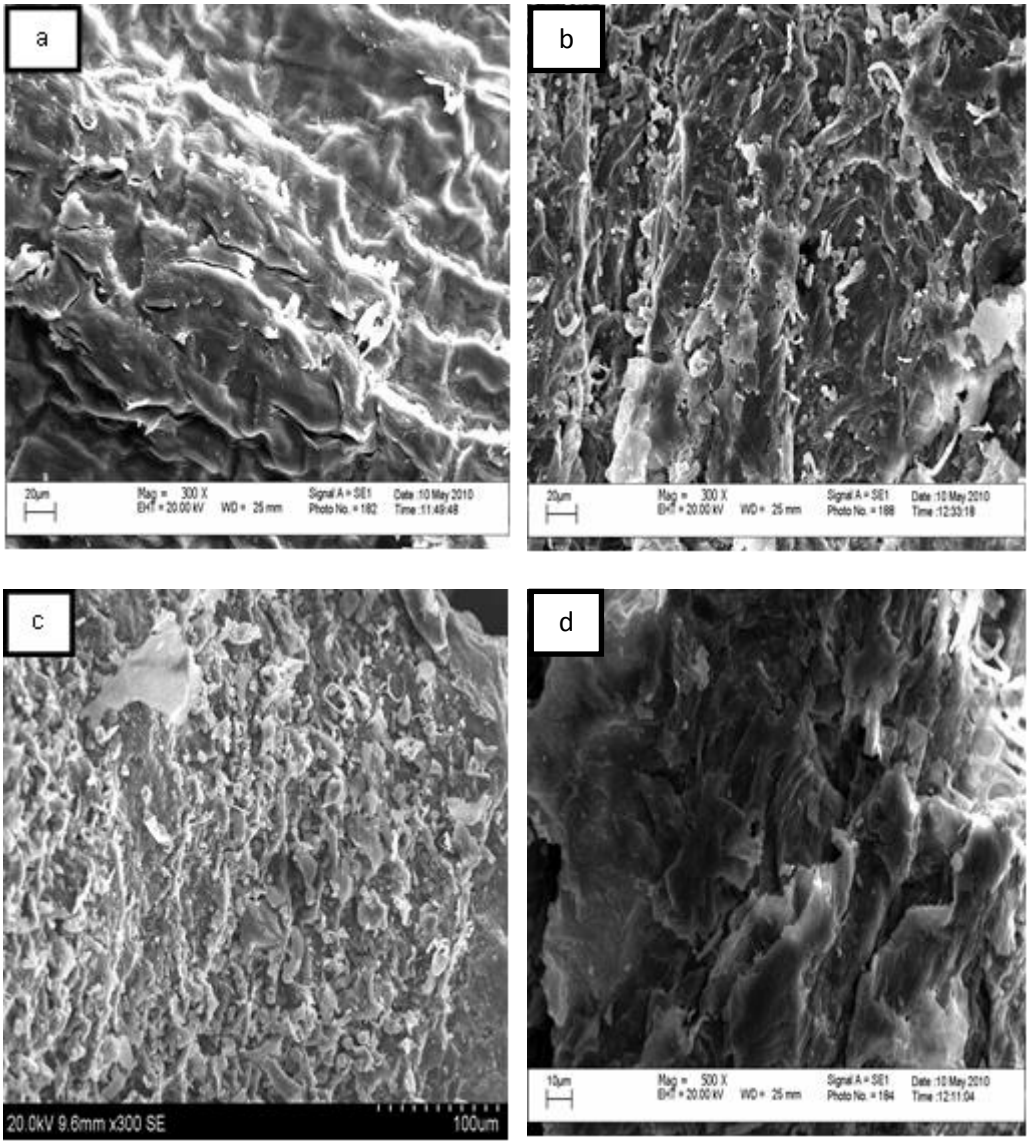


Symbols: a, 1 cm; b, 850 µm to 1 mm; c, 600 µm to 850 µm;

d, 300 µm to 600 µm**.**

FIGURE 4.2 SEM OF H. POLYRHIZUS PERICARPS PRETREATED BY MECHANICAL AND HYDROTHERMAL COMMUNITION

4.3.3 SUGAR PRODUCTION FROM PRETREATED PERICARPS OF *H.POLYRHIZUS*

Enzymes are natural plant proteins that initiate certain chemical reactions. For an enzymatic process acting on lignocelluloses to be efficient, pretreatment is

needed to rupture the crystalline structure of the lignocelluloses. Pretreatments, by facilitating the removal of lignin, can ‘open up’ cellulose and hemicelluloses structures. The reported components of pitaya biomass dictate that a combined hydrothermal and mechanical pretreatment is the most suitable for sugar production. Enzymatic hydrolysis processes have the advantages of high efficiency, controllable by-products, low cost materials, and relatively low energy usage (Aisan et al., 1997).

Enzymatic hydrolysis experiments were carried out on pretreated H. polyrhizus pericarp to determine the effects of pretreatments on the subsequent sugar yield (Table 4.2). For mechanical pretreatment alone the fermentable sugar yield was highest at 2.126 g/L for 1 cm cut pericarp, and lowest at 1.943 g/L for the 300 µm to 600 µm range (Table 4.2). All experiments recorded the highest sugar content at 16 h, except the pericarp size range 850 µm to 1 mm, which hit a lower, earlier peak.

The enzyme hydrolytic conversion fraction for autoclaved samples ranged from 31.52 % to 50.32 %, from smallest particle sizes (300 µm to 600 µm) to the largest (1 cm) (Table 4.3). The lowest sugar yield was recorded for the most severely communited (300 µm to 600 µm) samples, and it is possible that the severely altered structure inhibited the hydrolysis reaction, or a Maillard reaction was taking place due to the high protein content (Taherzedah and Karimi, 2007). Among the 1 cm cut pericarp, the non-autoclaved samples (Table 4.2) had a significantly lower sugar yield (0.243 g/g) compared to the autoclaved samples (0.381 g/g) (Table 4.3), demonstrating that autoclaving improves sugar yields from H. polyrhizus pericarp. Improvements in

enzymatic hydrolysis can be linked to xylan solubilisation, which is the main mechanism during hydrothermal pretreatment. It has been reported that xylan removal advantages the enzymatic digestion of cellulose in pretreated biomass (Kabel et al., 2007). The hydrolysis reaction profiles shown in Figures 4.3 and 4.4 contrast the synergistic effects between pretreatments.

As observed from Figure 4.3, more reducing sugar was released from 1 cm cut pericarps in comparison to the grinded smaller size particles Silva et al. (2010) reported that non-homogenous reduction behaviour could be observed in certain finely ground biomass particles, which was believed to greatly affect the final sugar yield. Another study by Bridgeman et al. (2007) supported the claim that small particle sizes lower cellulose content compared to larger particle sizes. The current study also found extensive communition to cause significant organic compound losses, leading to lower fermentable sugar yields. According to Chouchene et al. (2010), sample sizes below 0.5 mm released a large amount of volatile matters. An advantage of larger particle sizes is a reduction is the amount of residual ash left over as solid waste (Vanmvuka et al., 2003). The degradation of hemicellulose and cellulose is sped up by fine communition. Additionally, cut pericarp costs less to process in terms of both energy and mechanical overheads, leading to better economic scalability.

During hydrothermal pretreatment, the biomass samples were subjected to autoclaving at high temperature and pressure (121 ºC at 15 psi) for 15 min. Hemicellulose can be solubilised through hydrothermal pretreatment to make the cellulose more accessible for enzymatic hydrolysis (Prasad et al., 2006).

The water in the biomass rapidly decompresses which disrupts the plant cell wall. However, temperatures exceeding 220 ºC during a 2 min pretreatment caused recondensation and precipitation of soluble lignin and hemicellulose, resulting in the formation of furfural (Ramos, 2003). Other effects such as the breakdown of the lignocellulosic structure and the depolymerisation of the lignin components can also occur during hydrothermal pretreatment (Prasad et al., 2006).

Combined mechanical and hydrothermal pretreatment is preferred, as a single pretreatment is sometimes insufficient to enhance enzymatic digestibility (Chang et al., 2001). This is supported by the present study as the combined pretreatment of 1 cm cut pericarp significantly improved sugar yields (Table 4.3).

TABLE 4.2 YIELD AND PRODUCTIVITY OF SUGAR FROM PERICARPS PRETREATED WITH MECHANICAL COMMUNITION ALONE

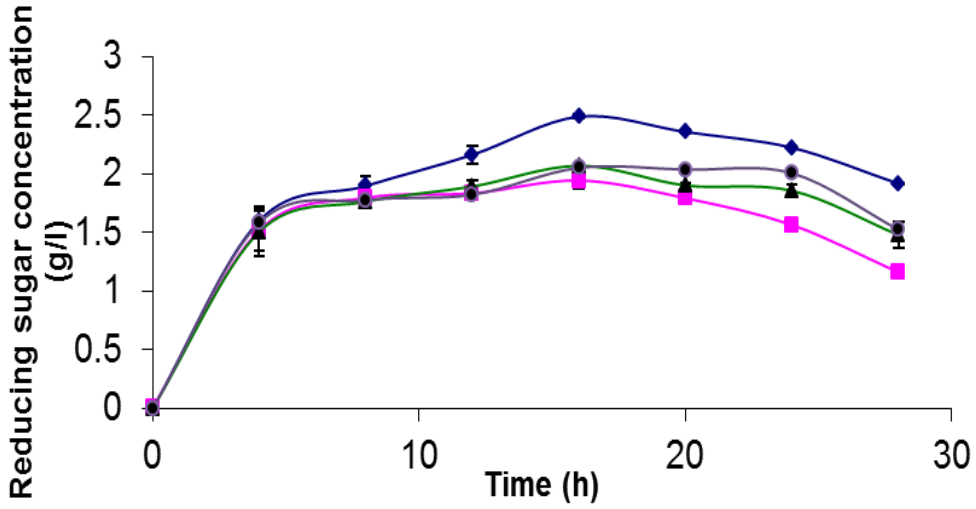
Size of particle	S_m (g/L)	t_m (h)	Yield (g/g)	Sugar productivity (g/L/ h)	Fractional hydrolytic conversion
1 cm	2.488 [b]	16	0.243 [b]	0.13 [c]	35.09 [b]
850 µm to 1 mm	2..020 [c,d]	12	0.202 [e]	0.17 [b]	31.42 [g]
600 µm to 850 µm	2.051 [c,d]	16	0.204 [d]	0.13 [d]	32.58 [d]
300 µm to 600 µm	1.943 [d,e]	16	0.193 [f]	0.12 [e]	34.91 [c]

Mean values in the same column with the same letters are not significantly different
S_m: Maximum sugar content (g/L)
t_m: Time taken to reach maximum sugar content (h)

TABLE 4.3 YIELD AND PRODUCTIVITY OF SUGAR FROM PERICARPS BY COMBINATION OF MECHANICAL COMMUNITION AND HYDROTHERMAL

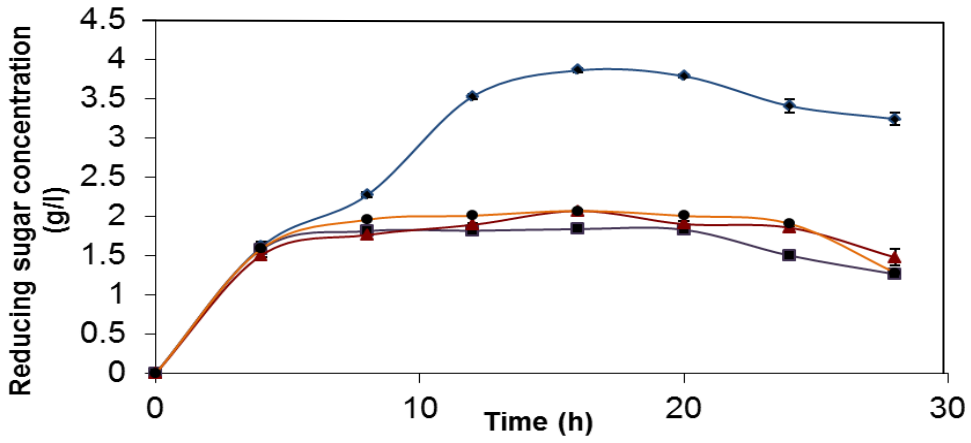
Size of particle	Sm (g/L)	t_m (h)	Yield (g/g)	Sugar productivity (g /L/ h)	Fractional hydrolytic conversion
1 cm	3.861 [a]	16	0.381 [a]	0.24 [a]	50.32 [a]
850 µm to 1 mm	2.069 [c]	16	0.206 [c]	0.13 [d]	31.34 [h]
600 µm to 850 µm	2.070 [c]	16	0.206 [c]	0.13 [d]	31.93 [e]
300 µm to 600 µm	1.838 [e]	16	0.183 [g]	0.12 [e]	31.52 [f]

Mean values in the same column with the same letters are not significantly different
S_m: Maximum sugar content (g/L)
t_m: Time taken to reach maximum sugar content (h)



Symbols: (♦), 1 cm; (▲), 850 µm to 1 mm; (●), 600 µm to 850 µm; (■), 300 µm to 600 µm. Error bars signify the mean ±standard deviation of three experiments. For data points without error bars, the errors were smaller than the size of the symbols.

FIGURE 4.3 ENZYMATIC HYDROLYSIS OF H. POLYRHIZUS PERICARP PRETREATED WITH MECHANICAL COMMUNITION ALONE



Symbols: (♦),1 cm; (▲), 850 µm to 1 mm ; (●), 600 µm to 850 µm; (■), 300 µm to 600 µm. Error bars signify the mean ±standard deviation of three experiments. For data points without error bars, the errors were smaller than the size of the symbols.

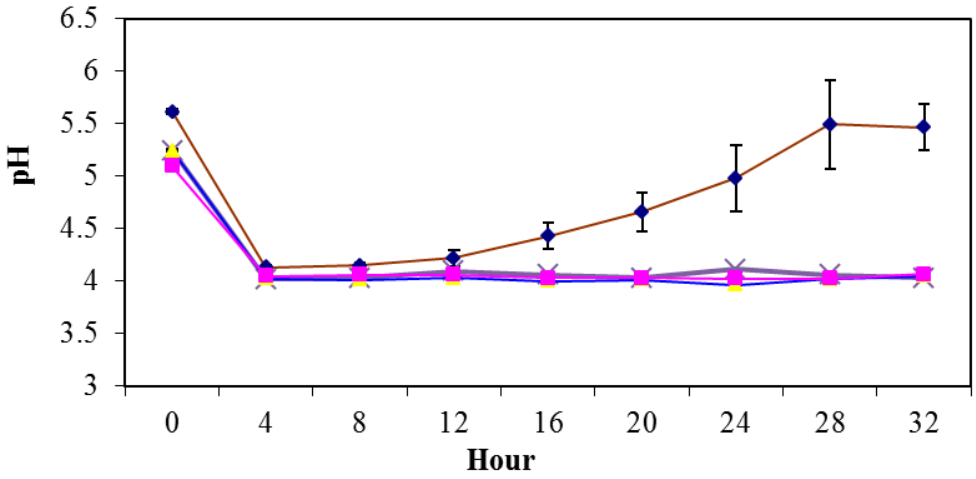
FIGURE 4.4 ENZYMATIC HYDROLYSIS OF H. POLYRHIZUS PERICARP PRETREATED WITH COMBINED HYDROTHERMAL AND MECHANICAL COMMUNITION AT 121 °C FOR 15 MIN

Various end products can be implied by investigating the pH level of the broth. Figures 4.5 and 4.6 compare the pH profiles for different particles sizes and pretreatments. The pH of the broth fluctuated between pH 5.1 and pH 5.6, which is very close to the optimum pH for these yeast systems. The pH is an important factor in hydrolysis, as low pH (pH 5 or lower) results in a higher yield of xylose (Biely, 1985). This is because a low pH medium promotes celluclast activity. When there is a low pH, an acetate group is released during the enzymatic hydrolysis process which then combines with a hydrogen ion to form acetic acid. The presence of acetyl xylan esterase in enzyme mixtures, produced by different xylanolytic fungi, has been reported previously (Biely, 1985). These esterases act in synergy with xylanase to break down xylan structure. Esterases are specific to acetylated xylan.

In both pretreatment scenarios, the pH of the broth decreased slightly during the first 4 h of hydrolysis due to the formation of acidic compounds. Enzymatic hydrolysis also releases acetate, but fortunately enzyme activity is only slightly affected by acetate (Maloney et al, 1985). Enzymatic hydrolysis operates most efficiently under conditions of low temperature, pH and pressure.

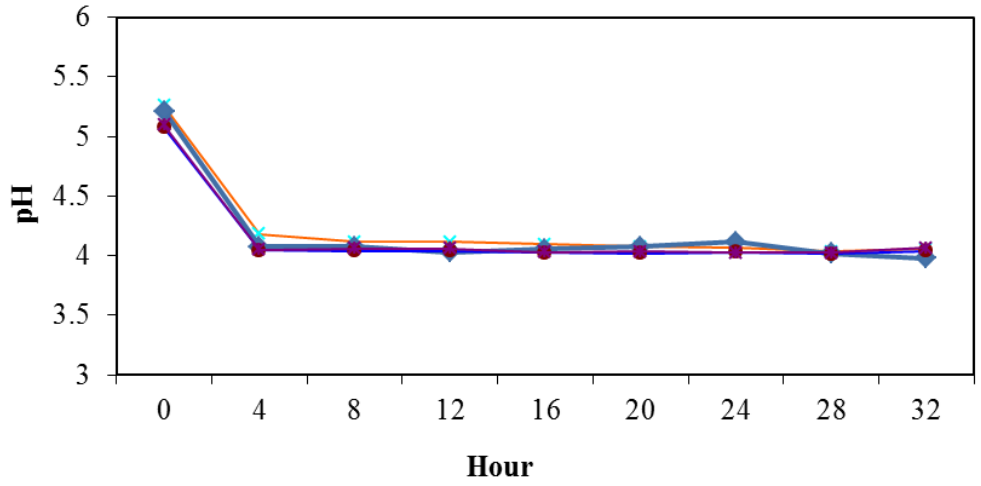
Different plants and plant parts vary in subunits, composition, polymer components, and concentrations of hemicelluloses. The probability of the formation of intermolecular hydrogen bonds and microfibrils is lowered by the absence of a primary alcohol group. Lacking intermolecular hydrogen bonds among the polysaccharide chains, xylan does not form a crystalline structure. Furthermore, uronic acids in the branches make xlyan an acidic

polysaccharide (Robyt, 1997). The complete breakdown of xylan involves several enzymes with varied specificity due to the complexity of the chemical structure of xylan. The mixture of enzymes is typically endo-1, 4-β-xylanase, β-D-xylosidase, acetyl xylan esterase, α-glucuronidase and arabinose (Aisan et al., 1997). This might explain why the pH of the broth decreases at the beginning of the hydrolysis process, as xylan breaks down to become an acidic polysaccharide.



Symbols: (♦),1 cm; (x), 850 µm to 1 mm ; (▲), 600 µm to 850 µm; (■), 300 µm to 600 µm. Error bars signify the mean ± standard deviation of three experiments. For data points without error bars, the errors were smaller than the size of the symbols.

FIGURE 4.5 AVERAGE PH PROFILE OF H. POLYRHIZUS PERICARP DURING ENZYMATIC HYDROLYSIS AFTER MECHANICAL COMMUNITION ALONE



Symbols: (x), 1 cm; (♦), 850 µm to 1 mm; (●), 600 µm to 850 µm; (*), 300 µm to 600 µm. Error bars signify the mean ± standard deviation of three experiments. For data points without error bars, the errors were smaller than the size of the symbols.

FIGURE 4.6 AVERAGE PH PROFILE OF H. POLYRHIZUS PERICARP DURING ENZYMATIC HYDROLYSIS AFTER COMBINED HYDROTHERMAL AND MECHANICAL COMMUNITION

4.4 Conclusions

The general aim of pretreatment was to overcome lignocellulose recalcitrance in the conversion of biomass to ethanol. The 1 cm cut pitaya with hydrothermal pretreatment had the highest hemicelluloses (29.28 %) and cellulose (46.47 %) contents, and the total organic content was significantly higher than for mechanical pretreatment alone.

Following pretreatment, the pitaya peel underwent enzymatic hydrolysis to convert polysaccharides into monomeric sugars. The present study concludes that the pretreatment by communition to 1 cm pericarp, combined with hydrothermal conditioning, enhanced the efficiency of enzymatic hydrolysis.

The maximum content and productivity of fermentable sugar recorded was 3.861 g/L and 0.24 g/L/h respectively for the combined hydrothermal and mechanical pretreatment of 1 cm pericarp.

CHAPTER 5

BIOETHANOL PRODUCTION USING SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

5.1 Introduction

Irrespective of the raw material used, the main steps involved in the production of ethanol are saccharification (breaking down of complex carbohydrates into monosaccharides in the presence of an enzyme), fermentation (conversion of the fermentable monosaccharides into ethanol and CO_2), and distillation (separation and drying to separate ethanol from by-products to make fuel grade ethanol). Sometimes saccharification and fermentation occur together, and this is known as Simultaneous Saccharification and Fermentation (SSF). SSF reduces the inhibition of saccharification by glucose, and the chances of bacterial contamination caused by the transfer of the medium to a new vessel. SSF is an effective process for the direct conversion of starch to ethanol (Ljunggren, 2005). The major advantage of the SSF process is that the yield of ethanol can be improved because ongoing fermentation converts the sugars that inhibit the formation of more sugar by hydrolysis. Other advantages include: a) a lower reactor volume, as only one reactor is necessary, b) lower enzyme requirement, and c) lower maintenance and requirement costs, since the process time is shorter (Prasad et al 2006, Sun and Cheng, 2002). The efficiency of this process depends upon the characteristics of the biomass, the quality of the enzyme used for saccharification (enzymatic hydrolysis), and the performance of the

yeast used for fermentation. Recent optimisations have been studied to minimize the number of necessary experiments, and to provide more information on the effects of study variables and interaction effects.

The main aim of this study was to maximize the bioethanol production by *Saccharomyces cerevisiae* from pitaya pericarp. Early optimisations included identifying factors such as incubation temperature, enzyme loading of cellulase and pectinase, loading of pretreated pitaya pericarp, initial pH of the medium, and the size of the inoculum. Response Surface Methodology (RSM) based on Central Composite Design (CCD) was then applied to the design model, to determine the optimum conditions for the fermentation process.

5.2 Materials and Methods

5.2.1 PREPARATION OF SAMPLES

Pitaya waste peel from frozen (-20 °C) storage, was communited and pretreated to sample sizes ranging from 1 cm to 300 µm. Samples were dried overnight at 60 °C in a fan forced oven to correct the moisture content before SSF was carried out.

5.2.2 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS (SSF)

Upon identification of a suitable pretreatment method to release the highest fermentable sugar concentration after enzymatic hydrolysis, SSF trials were conducted. Dry pretreated pericarp of different particle sizes were supplemented with: yeast extract, 10 g/L; and peptone, 20 g/L; in 250 mL conical flasks and autoclaved for 15 min at 121 °C. Pectinase (Pectinex Ultra SP) and cellulase (Celluclast 1.5 L) were then added to the flasks. The flasks were inoculated with *Saccharomyces cerevisiae* and fermented under the conditions determined by the statistical model. During the fermentation process, 3 mL of culture broth was taken at pre-determined time intervals and frozen for later analysis. The fermented broths were centrifuged at 9000 rpm and the supernatant used for analysis of fermentable sugar and ethanol concentration.

5.2.3 ANALYTICAL PROCEDURES

Soluble fermentable sugars were measured using the DNS acid method. The samples were then cooled in an ice-bath to prevent the evaporation of ethanol. The soluble ethanol concentration was measured by using GC on a capillary column (Zebron ZB-Wax plus), with detection by a flame ionization detector. Ethanol was gauged by retention time and calculated using an ethanol standard curve.

5.2.4 STATISTICAL EXPERIMENTAL DESIGNS

Plackett-Burman Experimental Design

The main factors affecting optimisation targets were screened for on all six critical variables using a Plackett-Burman design (Plackett and Burman, 1946). The software used to select experimental designs was Design-Expert (STAT-EASE Inc, Minneapolis, USA, Version 7.1.3). The Plackett-Burman design involved two levels, high (H) and low (L). These levels define the upper and lower limits of the range covered by each variable. Therefore, in this study a total of twelve runs were used for the six test variables, with each run duplicated (Table 5.2). The values of the coded levels of the independent variables used in the experiments are listed in Table 5.1. The standard error (SE) of the concentration effect was the square root of the variance of the effect. Equation 5.1 calculates the variance among the variables.

$$V_{eff} = \sum(X_d)^2 / n$$

EQUATION 5.1

where, V_{eff} is the variance of the concentration effect, X_d is the concentration effect for the variable, and *n* is the number of variables.

The significance level (p-value) was calculated using Student's t-test (Equation 5.2).

$$p - value = E(xi)/SE$$

EQUATION 5.2

where = *E (Xi)* is the effect of the variable X_i.

TABLE 5.1 EXPERIMENTAL RANGE AND LEVELS OF INDEPENDENT VARIABLES IN THE PLACKETT-BURMAN DESIGN

Variable	Level		
	-1	0	1
Incubation temperature (°C)	35	40	45
Cellulase loading (mL/ g)	0.05	0.7	1.5
Pectinase loading (mL/g)	0.05	0.7	1.5
Substrate loading (g/L)	50	75	100
Initial pH	4.8	5.4	6
Innoculum size (%v/v)	3	6.5	10

TABLE 5.2 PLACKETT-BURMAN DESIGN MATRIX USING CODED VALUES FOR 6 INDEPENDENT VARIABLES

Run No	Factors (Coded Values)					
	*A (°C)	B (mL/g)	C (mL/g)	D (g/L)	E (pH)	F (%v/v)
1	-1	1	1	1	-1	-1
2	1	-1	1	1	1	-1
3	-1	1	1	-1	1	1
4	1	1	-1	-1	-1	1
5	1	1	1	-1	-1	-1
6	-1	-1	1	-1	1	1
7	-1	1	-1	1	1	-1
8	1	1	-1	1	1	1
9	-1	-1	-1	1	-1	1
10	1	-1	1	1	-1	1
11	1	-1	-1	-1	1	-1
12	-1	-1	-1	-1	-1	-1

*Symbol: A,Incubation temperature; B, cellulase loading; C, pectinase loading; D, substrate loading; E, intial pH; F, inoculum size

Path of Steepest Ascent

After the important variables had been identified, the region around the current operating conditions was explored. The method of steepest ascent given by Box and Wilson (1951) was carried out by moving sequentially in the

direction of the maximum increase in the response. This direction, called the path of steepest ascent, is given by

$$x_2 = \frac{b_2}{b_1}(x_1), \ \ldots\ldots, \ x_k = \frac{b_k}{b_1}(x_1),$$

where b_i,... ,b_k are estimates. Experiments were performed on the path of steepest ascent until no further increase in response was observed. The experimental design is explained further in Section 5.3.2.

Response Surface Methodology

RSM designs were then used to explore the regions thoroughly and obtain the point of maximum response. CCD was employed to determine variables from the screening experiment that were significant, and optimal levels were identified from RSM (Box et al., 1978; Rekab and Shaikh, 2005).

The three most significant factors, i.e. incubation temperature, cellulase loading and pectinase loading were investigated at five level (-1.682, -1, 0, 1, 1.682), and are discussed further in Section 5.3.3. All trials were performed in duplicate with the mean values of the maximum ethanol concentration as the response output.

5.2.5 DATA ANALYSIS

Analysis of variance (ANOVA) models were used on the experimental data, and coefficients computed using SAS ® Proprietary Software Release 6.12. The statistical analysis of the optimizations for the experimental data were

computed and experimental designs made using Design-Expert, Version 7.0 (STAT-EASE Inc., Minneapolis, USA)

5.3 Results and Discussion

Ethanol fermentation was performed by a simultaneous saccharification and fermentation (SSF) process in which the fermentable sugars formed in saccharification are concurrently fermented to ethanol. The SSF process reduces both the osmotic stress on yeast cells and the inhibition of hydrolysis enzymes (Wang et al, 1999). Inhibition of the end products of hydrolysis in the SSF process has been extensively studied (Takagi et al., 1977; Blotkamp et al., 1978; Szczodrak and Targonski, 1989; Saxena et al., 1992; Philippidis et al., 1993; Zheng et al., 1998). The fungus T.reesei and yeast S.cerevisiae are the common microorganisms used in SSF. The optimal temperatures for hydrolysis and fermentation are 45 °C to 50 °C, and 30 °C respectively, and the SSF process compromises to 38 °C (Philippidis, 1996). Hydrolysis is the rate limiting process in SSF (Phillipidis and Smith, 1995). In comparison to the separate hydrolysis and fermentation (SHF) process, SSF has the advantages of lower enzyme loading, increased hydrolysis rate, higher product yield, significantly lower processing time, and uses only a single reactor. SSF also has lower hygiene requirements, as glucose is removed by fermentation as it is produced. However, Wu and Lee (2007) found that some cellulose activity was sacrificed at 38 °C, which may explain some of the apparent inhibition of cellulase by ethanol in the SSF process. The disadvantages of SSF are conflicting hydrolysis and fermentation temperatures, and the inhibition of enzymes by ethanol.

5.3.1 PLACKETT-BURMAN DESIGN

The six factors of incubation temperature, cellulase loading, pectinase loading, initial pH, and inoculum size were selected from the literature as the main factors affecting the process. The Plackett-Burman design was used to evaluate the level of importance of the six selected factors as they affect ethanol production from pitaya pericarp.

Table 5.3 shows the experimental responses obtained from the twelve runs of the Placket-Burman tests. Each run was done in duplicate to ensure accuracy. The design enables good variation of the optimisation process. Table 5.3 indicates that there was close agreement between actual and predicted values of the maximum ethanol concentration produced. The maximum ethanol concentration and ethanol yield were both stipulated as response values for the Plackett-Burman design.

TABLE 5.3 PLACKETT-BURMAN DESIGN AND THE EXPERIMENTAL RESULTS

Runs	Incubation Temperature (°C)	Cellulase loading (mL/g)	Pectinase loading (mL/g)	Substrate loading (g/L)	pH	Innoculum size (%v/v)	Maximum ethanol concentration (g/L)		Time max EtOH (h)	Ethanol productivitiy (g/L/h)	Ethanol yield (g/g)
							actual	predicted			
1	35	1.5	1.5	100	4.8	3	10.7	10.5	24	0.45	12.3
2	45	0.05	1.5	100	6	3	0	-0.31	0	0	0
3	35	1.5	1.5	50	6	10	7.89	7.25	36	0.22	7.39
4	45	1.5	0.05	50	4.8	10	2.18	1.89	12	0.18	4.51
5	45	1.5	1.5	50	4.8	3	0	1.37	0	0	0
6	35	0.05	1.5	50	6	10	4.84	5.49	24	0.2	5.54

Table 5.3 Plackett-Burman design and the experimental results **(**Continued**)**

Runs	**Incubation temperature (°C)**	**Cellulase loading (mL/g)**	**Pectinase loading (mL/g)**	**Substrate loading (g/L)**	**pH**	**Innoculum size (%v/v)**	**Maximum ethanol concentration (g/L)**		**Time max EtOH (h)**	**Ethanol productivitiy (g/L/h)**	**Ethanol yield (g/g)**
							actual	**predicted**			
7	35	1.5	0.05	100	6	3	11.1	11.3	36	0.31	0
8	45	1.5	0.05	100	6	10	2.43	1.97	12	0.2	2.16
9	35	0.05	0.05	100	4.8	10	7.7	9.24	36	0.21	0.21
10	45	0.05	1.5	100	4.8	10	0.13	-0.66	12	0.01	1.59
11	45	0.05	0.05	50	6	3	0	0.48	0	0	0
12	35	0.05	0.05	50	4.8	3	11.1	9.51	24	0.46	0

The ANOVA tables for the maximum ethanol response (Table 5.4 and Table 5.5) indicate that of the six variables tested; only temperature, cellulase and pectinase loading had a significant effect on maximum ethanol produced at a confidence level of $p < 0.10$. The linear regression equation is expressed in Equation 5.3:

$$\ddot{Y} = 41.10661 - 0.80933x1 + 1.21379x2 - 1.24598x3 + 0.020267x4 - 0.77778x5 - 0.18333x6$$

EQUATION 5.3

where ÿ represents the response value of the maximum ethanol produced, and x1, x2, x3, x4, x5 , x6 denote the coded values of incubation temperature, cellulase loading, pectinase loading, substrate loading, initial pH and inoculum size respectively.

The results show that the regression model was significant with p-value = 0.0021. The adjusted R^2 was 0.92 (Table 5.4) which means 92 % of variation for the maximum ethanol produced was distributed in the regression equation, and only 8% of the variation could not be explained by the model. The R^2 of 0.96 (Table 5.4) indicates that there was a good fit between actual and predicted values of the maximum ethanol produced (Table 5.3). The coefficient value (Table 5.5) shows the correlation tendency of the main factors affecting ethanol produced. Within the test range, cellulase and substrate loading show positive correlation with the maximum ethanol produced. This means that maximum ethanol was produced when the upper limit of cellulase and substrate loading were chosen. In contrast, incubation temperature, pectinase loading, initial pH, and inoculum size showed negative

correlation (Table 5.5), meaning that ethanol product was maximised when the lower limit of each was selected.

The Student t-test, p-value, and other metrics are given in Table 5.5. The p-values indicate the significance of each variable. The smaller the p-value, the more significant the corresponding variable. Statistically significant differences were observe for incubation temperature, cellulase loading, and pectinase loading (p-value < 0.10).

Therefore, the main factors affecting the maximum ethanol produced using hydrothermally pretreated 1 cm cut pitaya pericarp were determined to be incubation temperature (p-value = 0.0001), cellulase loading (p-value = 0.0712), and pectinase loading (p-value = 0.0661).

TABLE 5.4 STATISTICAL ANALYSIS OF MODEL (ANOVA) USING MAXIMUM ETHANOL CONCENTRATION AS RESPONSE

Source	Sum of squares	Degree of freedom	Mean square	F-value
Model	226.23	6	37.7	21.15
Incubation temperature (°C)	196.51	1	196.51	110.25
Cellulase loading (mL/g)	9.29	1	9.29	5.21
Pectinase loading (mL/g)	9.79	1	9.79	5.49
Substrate loading (mL/g)	3.08	1	3.08	1.73
pH	2.61	1	2.61	1.47
Innoculum size (%v/v)	4.94	1	4.94	2.77

R^2 = 0.96 and adjusted R^2= 0.92

TABLE 5.5 PLACKETT-BURMAN DESIGN; THE EFFECTS OF INDEPENDENT VARIABLES BASED ON MAXIMUM ETHANOL CONCENTRATION PRODUCED

Term	Coefficient	Standard error	*t*-value	*p*-value
Incubation temperature	-4.05	0.39	10.5	0.0001
Cellulase loading	0.88	0.39	2.5	0.0712
Pectinase loading	-0.9	0.39	2.5	0.0661
Substrate loading	0.51	0.39	1.6	0.2457
pH	-0.47	0.39	1.5	0.2801
Innoculum size	-0.64	0.39	1.5	0.1568

Ethanol yield as response was investigated using the same Plackett-Burman design (Table 5.3). Analysis of variance (ANOVA) was used to test the significance and suitability of the model (Tables 5.6 and 5.7). The regression model was a good fit with a *p*-value of 0.0085. Adjusted R^2 was 0.85 (Table 5.6), meaning that 85 % of the variation for ethanol yield fit the regression equation. The first order regression equation was as shown below in Equation 5.4.

$$\ddot{Y} = 11.77996 - 0.27376x_1 + 0.63864x_2 - 0.56823x_3$$
$$+0.010890x_4 - 0.39409x_5 + 0.12242x_6$$

EQUATION 5.4

where $\ddot{Y}$ represents the response value of ethanol yield, and x_1, x_2, x_3, x_4, x_5 , x_6 denote the coded values of incubation temperature, cellulase loading,

pectinase loading, substrate loading, initial pH, and inoculum size respectively.

The $R^2 = 0.93$ (Table 5.6) value indicates a good fit of the model to the ethanol yield value.

The coefficient values (Table 5.7) indicate that cellulase loading, substrate loading and inoculum size had a positive effect on ethanol yield within the test scale. This implies that higher cellulase loading, substrate loading, and inoculum size are more suitable for increasing bioethanol production. Conversely, increasing incubation temperature, pectinase loading, and pH had a negative effect on ethanol yield.

The *p*-values indicate the significance of each variable. A *p*-value > 0.10 was observed for substrate loading, initial pH and inoculum size (Table 5.7), indicating that there is no significant effect due to these three factors within the test ranges. Statistically significant factors were incubation temperature (*p*-value = 0.0009), cellulase loading (*p*-value = 0.063), and pectinase loading (*p*-value = 0.0875).

Therefore, incubation temperature, cellulase loading, and pectinase loading were the main factors affecting ethanol yield. Regardless of maximum ethanol concentration or yield, the significant parameters affecting the fermentation process remained the same.

TABLE 5.6 STATISTICAL ANALYSIS OF MODEL (ANOVA) USING ETHANOL YIELD AS RESPONSE

Source	Sum of squares	Degree of freedom	Mean square	F-value
Model	30.86	6	30.86	11.35
Incubation temp. (°C)	22.48	1	22.48	49.6
Cellulase loading (mL/g)	2.57	1	2.57	5.68
Pectinase loading (mL/g)	2.04	1	2.04	4.49
Substrate loading (mL/g)	0.89	1	0.89	1.96
pH	0.67	1	0.67	1.48
Innoculum size (%v/v)	2.20	1	2.20	4.86

R^2= 0.93 and adjusted R^2= 0.85

TABLE 5.7 PLACKETT- BURMAN DESIGN; THE EFFECT OF INDEPENDENT VARIABLES BASED ON ETHANOL YIELD RESPONSE

Term	Coefficient	Standard error	*t*-value	*p*-value
Incubation temperature	-1.37	0.19	7.04	0.0009
Cellulase loading	0.46	0.19	1.8	0.063
Pectinase loading	-0.41	0.19	1.79	0.0875
Substrate loading	0.27	0.19	1	0.2202
pH	-0.24	0.19	1	0.2781
Innoculum size	0.43	0.19	1.79	0.0786

Effect of Individual Significant Parameters

The results of the screening experiments show that incubation temperature, cellulase loading, and pectinase loading had the strongest effect on enhancing

the fermentation process. Supplementation with cellulase enzyme had a positive effect on the maximum ethanol concentration and ethanol yield. Cellulase is a class of enzyme produced by fungi, bacteria and protozoa. Cellulase enzymes are important to fermentation as they catalyse the hydrolysis of cellulose by breaking down cellulose to beta-glucose (Barr et al., 1996). In the SSF process, as sugars are produced by enzymes, yeasts concurrently convert sugars to ethanol (Gauss. 1976).

Pectinase loading showed a negative correlation, indicating that lower concentrations of pectinase are favoured for ethanol production from pretreated pitaya pericarp. Pectin is a component in the primary cell wall of plant and helps to bind plant cells wall. The pectinase enzyme is used to degrade pectin which will cause the exposure of other cell wall components such as hemicellulose and cellulose. Pectinase has been reported to have degradation effect on polysaccharides in middle lamella. This degradation activity will cause by-products such as phenolic compounds, flavonoids, etc. Nattaporn and Pranee, 2011). Phenolic compounds negatively affect the growth and fermentation rates of some yeast strains (Zheng et al., 1998), explaining the negative correlation of increasing pectinase enzyme concentration on ethanol concentration and yield.

Temperature is an important factor in SSF because there is a large gap between the optimum temperature of saccharification and fermentation by yeast. Enzyme activity will increase with temperature until a critical denaturing threshold is reached (Xiongjun, 2007), but fermentation kinetics and yeast metabolism are negatively affected at much lower temperatures

(Varga et al., 2004). As expected, increasing incubation temperature had a negative effect (Table 5.5 and 5.7) on ethanol concentration and yield.

Six variables were used to screen for the main factors of bioethanol production using a Plackett-Burman design. Variables with a p-value < 0.10 (confidence level above 90%) were selected as the significant parameters from the ANOVA analysis (Table 5.5 and 5.7). Incubation temperature, cellulase loading, and pectinase loading were chosen for further optimization based on ethanol concentration and ethanol yield as responses in the Plackett-Burman.

5.3.2 SINGLE RESPONSE: PATH OF STEEPEST ASCENT

The calculation of the path of steepest ascent is an important part of response surface methodology (RSM). The direction of the steepest ascent path must be determined precisely by calculation. A first order model was used to determine the settings for the next experiment, moving towards optimum conditions.

The model for the current operating conditions in terms of the coded variables is:

$$y = b_0 + b_1x_1 + b_2x_2 + \ldots\ldots + b_kx_k$$

EQUATION 5.5

where y represents the yield, and x_1 and x_2 are the predictor variables. This problem may be solved with the help of an optimization solver, but in the current context this was unnecessary, as the solution is a simple equation of variable coordinates on the steepest ascent path, separated from the origin by the distance p, given by:

$$x_i = \frac{p.\, b_i}{\sqrt{\sum_{j=1}^{k} \binom{k}{j} b^2}} \quad \text{where } p \leq 2$$

EQUATION 5.6

This equation provides better parameter estimates and therefore a more consistent search direction. To maximise *y*, *p* = 1 was selected since a point on the steepest ascent direction distanced one unit (coded units) from the origin was desired. For instance, a decrease in temperature of 5 °C requires cellulose loading to be varied by 0.15 mL/g, and pectinase loading by -0.15 mL/g, as shown in Table 5.8.

TABLE 5.8 EXPERIMENTAL VALUES USING PATH OF STEEPEST ASCENT

Incubation temperature (°C)	Cellulase loading (mL/g)	Pectinase loading (mL/g)	Maximum fermentable sugar concentration (g/L)	Maximum ethanol concentration (g/L)	Ethanol yield (g/g)
30	1.075	0.475	2.92	3.51	1.202
35	0.925	0.625	5.35	6.05	1.13
40	0.775	0.775	1.5	1.69	1.13

From Table 5.8, the optimum temperature was 35 °C, with increased cellulase loading to 0.925 mL/g, and decreased pectinase loading to 0.625 mL/g. Regression models were then applied to find the optimum conditions for the highest concentration of ethanol.

As the screening for both the maximum ethanol concentration and ethanol yield resulted in the same significant parameters, an experimental design using

RSM was developed by using maximum ethanol concentration as the main evaluation criteria to optimize the conditions for bioethanol production.

5.3.3 EXPERIMENTAL DESIGN USING RESPONSE SURFACE METHODOLOGY (RSM)

The preceding statistical analysis showed that ethanol concentration was greatly affected by temperature and enzyme dosages. RSM is generally employed to study multivariable effects, and to find optimal conditions for systems (Sriroth, 2006). Central Composite Design (CCD), which has equal predictability in all directions from the centre, and is usually well optimised for fitting quadratic models, is the most common experimental design used in RSM (Ohta and Hayashida, 1983).

Table 5.9 shows the ranges and levels of the three significant variables in the CCD experiments selected on the basis of the Plackett Burman design and path of steepest ascent tests. The maximum bioethanol concentration was selected as the response.

Therefore, a 2^3 factorial design was chosen with six star points ($\alpha \pm 1.682$) and two centre points. The variables were coded as

$$x1 = \frac{xi - x * i}{\Delta xi}$$

where x_i is the coded value of the i th test variable, X_1 the actual value of the i th test variable, X^*_i the value of X_i at the centre of the investigated area, and ΔX_i the step size in the regression equation.

TABLE 5.9 INDEPENDENT VARIABLES IN THE EXPERIMENTAL RSM PLAN

Variables	**coded levels**				
	-1.682	-1	0	1	1.682
Temperature, *X*1 (°C)	28.59	32	37	42	45.41
Cellulase, *X*2 (mL/g)	0.67	0.78	0.93	1.08	1.18
Pectinase, *X*3(mL/g)	0.37	0.47	0.63	0.78	0.88

TABLE 5.10 EXPERIMENTAL DESIGN BASED ON CCD WITH EXPERIMENTAL AND PREDICTED VALUES OF BIOETHANOL PRODUCTION

Run	Incubation temperature (°C)	Cellulase loading (mL/g)	Pectinase loading (mL/g)	Maximum ethanol concentration (g/L)	
				Actual	Predicted
1	32	0.78	0.47	7.30	7.26
2	37	0.93	0.63	4.60	4.45
3	42	1.08	0.78	5.00	5.25
4	42	0.78	0.78	2.90	2.99
5	32	0.78	0.78	6.48	6.51
6	37	0.93	0.63	5.44	5.47
7	32	1.08	0.47	4.20	4.33
8	42	0.78	0.47	3.70	3.83
9	37	0.93	0.63	7.40	7.27
10	37	0.93	0.63	7.30	7.27
11	42	1.08	0.47	7.10	7.27
12	32	1.08	0.78	7.10	7.27
13	37	1.18	0.63	5.80	6.00
14	37	0.67	0.63	3.15	3.22
15	45	0.93	0.63	7.44	7.34
16	37	0.93	0.63	4.33	4.27
17	37	0.93	0.37	4.80	4.96
18	37	0.93	0.63	5.10	5.04
19	37	0.93	0.88	7.40	7.27
20	28	0.93	0.63	7.30	7.27

Actual values of statistically selected variables, along with the predicted and actual responses, presented in Table 5.10. The experimental results fit a second-order polynomial equation.

$$Y = -40.62899 + 1.79187x_1 + 18.61264x_2 + 35.28442x_3 + 0.26333x_1x_2 + 0.48667x_1x_3 - 6.44444x_2x_3 - 0.033498x_1^2 - 15.77080x_2^2 - 37.92682x_3^2$$

EQUATION 5.7

where Y represents the maximum ethanol concentration (g/L); x1, x2, x3 represent incubation temperature, cellulase loading, and pectinase loading

respectively. Positive terms have synergetic effect, while negative terms have an antagonistic effect.

The validity of the fitted model was evaluated and its ANOVA for the response surface of the quadratic model is shown in Table 5.11. Statistical significance was determined by F-test (Table 5.11). From the ANOVA, the model is significant at the 95 % confidence level, with F-value of 11916.37 and p-value < 0.0001.

The coefficients R^2 and adjusted R^2 represent the model fitting reliability (Lichts, 2008). R^2= 0.9999 suggests that almost 99.99 % of the variance was attributed to the variables, and that only 0.01 % of the total variations cannot be explained by the model. The closer R2 is to one, the stronger the model, and the better the response prediction (Chauhan et al, 2006). Adjusted R^2 = 0.9998 implies a very good adjustment of the quadratic model to the experimental data. The adequacy of the quadratic model was also supported by the excellent agreement between actual and predicted values of the response (maximum ethanol concentration), as shown in Table 5.10.

The coefficient of the variation (CV) is indicative of greater precision and reliability when the value is low. The CV was 0.30 %.

The signal to noise ratio was measured by 'Adeq Precision' (Table 5.11). A ratio greater than 4 is preferred, and the measured ratio of 295.393 indicates that the signal is substantially greater than the noise. This model is reliable and can be used to navigate the design space due to this high signal fidelity.

The lack-of-fit F-value = 3.42, and implies that the model is a good fit to the experimental data (Table 5.11). The associated p-value of 0.1019 assures that there is only a 10.19 % chance that a lack-of-fit F-value this large could occur due to noise. If the p-value of the lack-of-fit test were significant ($p < 0.05$), it would indicate that a more complicated model is necessary (Banik et al, 2007).

TABLE 5.11 ANOVA FOR MAXIMUM ETHANOL PRODUCTION USING CODED VALUES

Source	SS	d.f	MS	F-value	Prob(P)>F
Model	34.73	9	3.86	11916.37	<0.0001
X1	6.62	1	6.62	20434.31	<0.0001
X2	7.22	1	7.22	22302.36	<0.0001
X3	1.9	1	1.89	5.86	0.0361
X1X2	0.31	1	0.31	963.72	<0.0001
X1X3	1.07	1	1.07	3291.58	<0.0001
X2X3	0.17	1	0.17	519.46	<0.0001
X12	10.11	1	10.11	31213.53	<0.0001
X22	1.81	1	1.81	5604.08	<0.0001
X32	10.49	1	10.49	32410.79	<0.0001
Residual	3.238	10	3.238		
Lack of fit	2.505	5	5.009	3.42	0.1019
Pure error	7.333	5	1.467		
Total	34.73	19			

R2= 0.9999, adjusted R2= 0.9998, predicted R2= 0.9994
C. V. %= 0.30
Adeq Precision= 295.393
SS: sum of squares
d.f: degrees of freedom
MS: mean square
X1= temperature, °C
X2= cellulase enzyme, mL/g
X3= pectinase enzyme, mL/g

The relationship between predicted and experimental values of maximum ethanol concentration can be seen in Figure 5.1. There is a high correlation

(R^2 = 0.9988) between the experimental and predicted values. The experimental data gives a good estimate of the response of the system within the range studied.

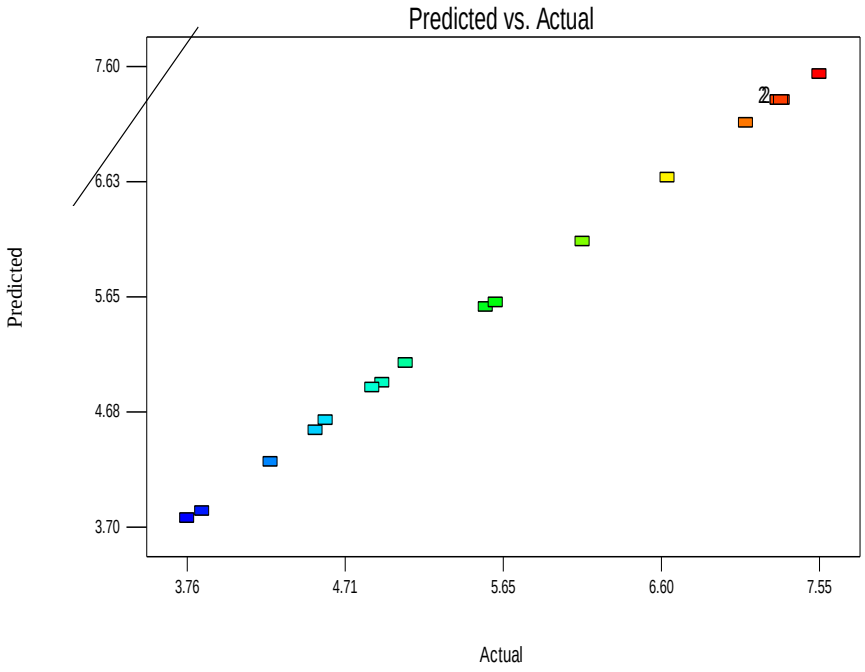


FIGURE 5.1 EXPERIMENTAL VALUES VERSUS PREDICTED VALUES FOR RSM MODEL

The normal probability plots of studentized residuals for maximum ethanol concentrations are shown in Figure 5.2. If the residuals follow a normal distribution, then the points will plot in a straight line. Hence, although some expected scattering can be seen in Figure 5.2, the data is normally distributed. The normal probability plot (Figure 5.2) confirms the validity of the quadratic model approximation.

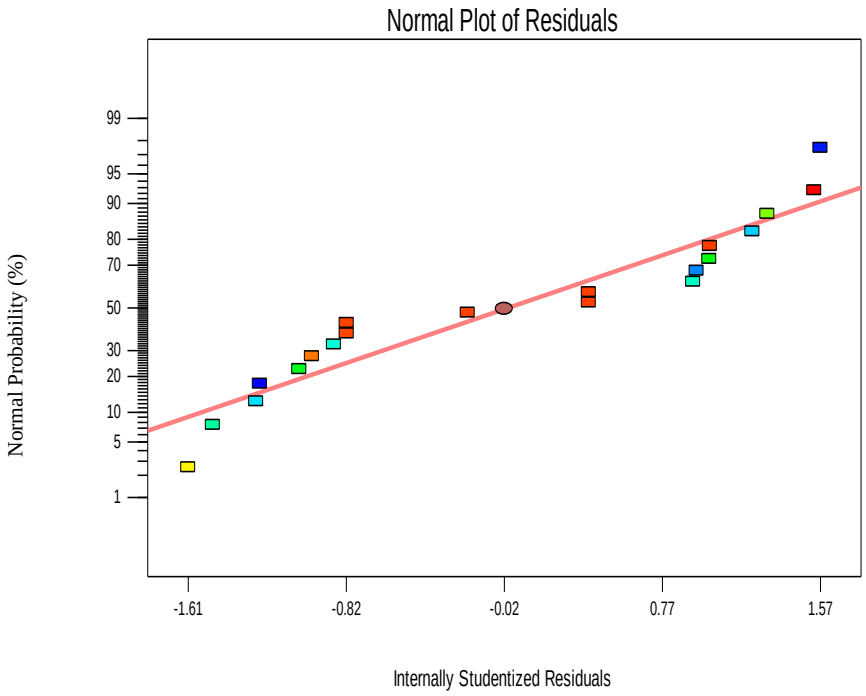


FIGURE 5.2 NORMAL PROBABILITY PLOT OF STUDENTIZED RESIDUALS

Figure 5.3 plots residual versus predicted values for maximum bioethanol concentrations. The points are scattered randomly within the range of the residuals, with no obvious pattern or uncommon structure, confirming that the observed runs were independent, and that no violation of the constant variance assumption was observed. The studentized residuals versus run (Figure 5.4) shows randomly scattered points ranged between ± 1.5 which means the errors were normally distributed and insignificant.

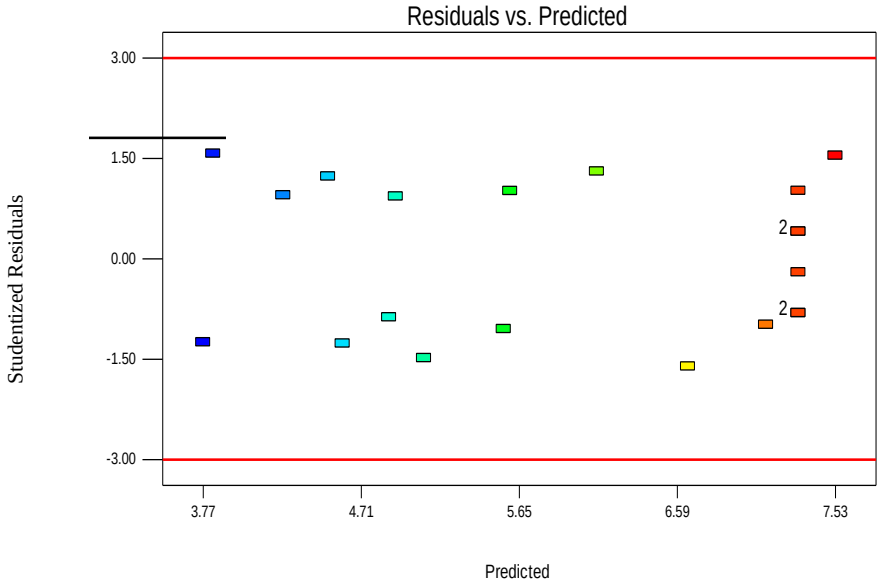


FIGURE 5.3 DIAGNOSTIC PLOT OF STUDENTIZED RESIDUALS VERSUS PREDICTED VALUES FOR MAXIMUM ETHANOL CONCENTRATIONS

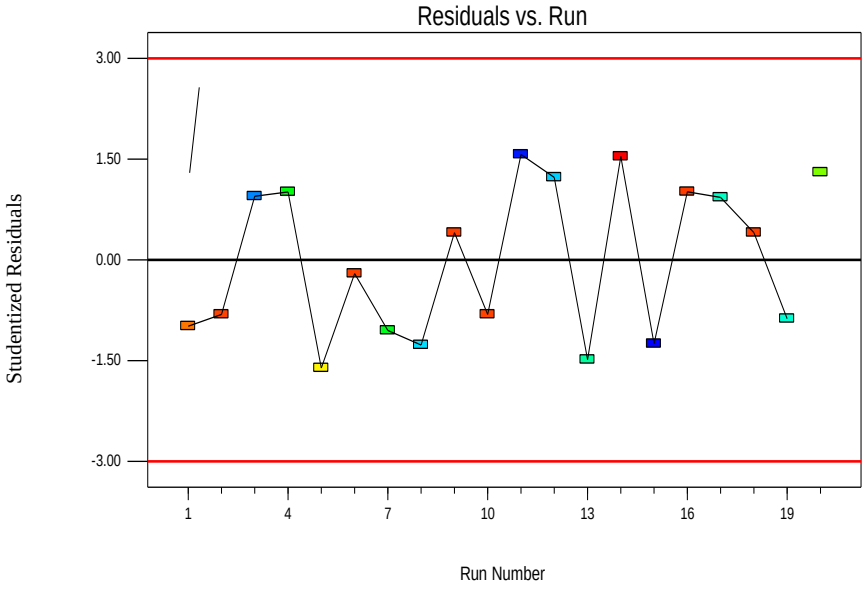
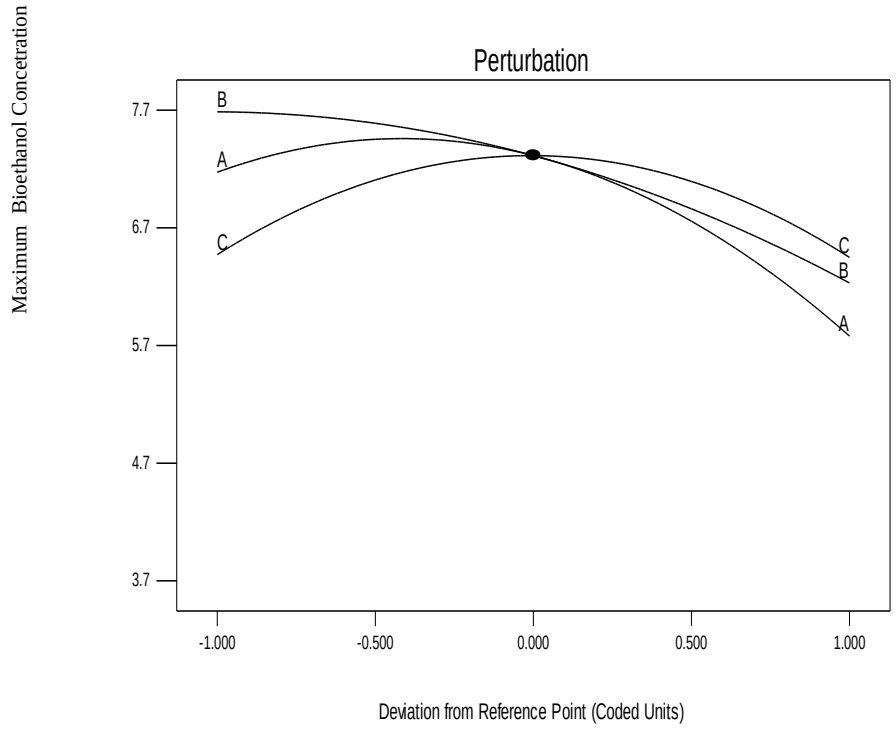


FIGURE 5.4 DIAGNOSTIC PLOT OF STUDENTIZED RESIDUALS VERSUS RUN NUMBER FOR MAXIMUM ETHANOL CONCENTRATIONS

Figure 5.5 shows the perturbation graph. The perturbation graph revealed the comparative effects of all independent variables on maximum bioethanol concentration. The curvature of the incubation temperature, cellulase loading,

and pectinase loading plots show that the response (maximum ethanol concentration) was sensitive to these variables.



Symbols: A, incubation temperature (°C); B, cellulase loading (mL/g); C, pectinase loading (mL/g).

FIGURE 5.5 PERTURBATION PLOT FOR MAXIMUM ETHANOL CONCENTRATION

Interaction among Factors Influencing Bioethanol Production

The RSM was used to investigate the interactions between variables, and shown in Figure 5.6. The variables in this CCD model were incubation temperature, cellulase loading, and pectinase loading.

Figure 5.6 (a) shows the effects of varying incubation temperature and cellulase loading, at constant pectinase loading (0.69 mL/g), on maximum ethanol concentration. As incubation temperature increases above 37.0 °C, the concentration of bioethanol decreases. The maximum bioethanol concentration

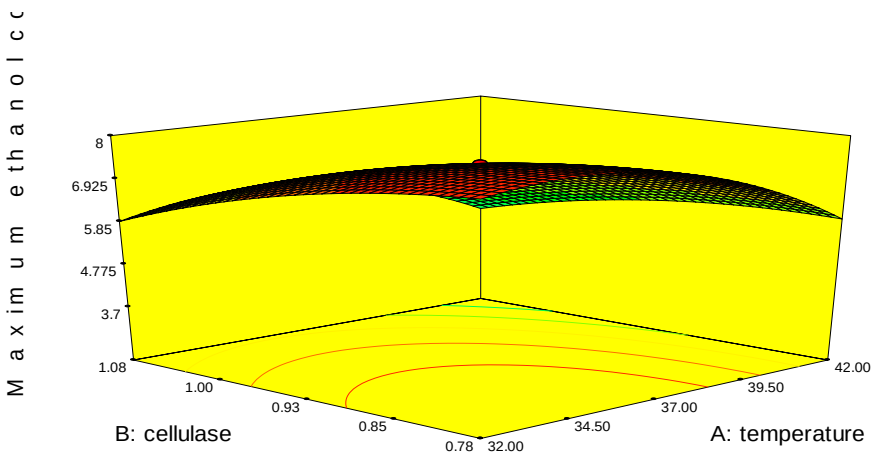
was achieved at an incubation temperature of ~35 °C. Temperature is an important factor in SSF because of the difference in optimum temperature of saccharification (50 °C) and that of yeast fermentation (35 °C). Temperatures above 37 °C limit S. cerevisiae growth and ethanol production.

Figure 5.6 (b) represents the cooperative effect of pectinase loading and incubation temperature, at constant cellulase loading (0.85 mL/g), on maximum ethanol concentration. As pectinase loading increases above ~0.7 mL/g, the concentration of bioethanol begins to decrease. Maximum bioethanol production ~7.6 g/L was obtained at an incubation temperature of ~35 °C.

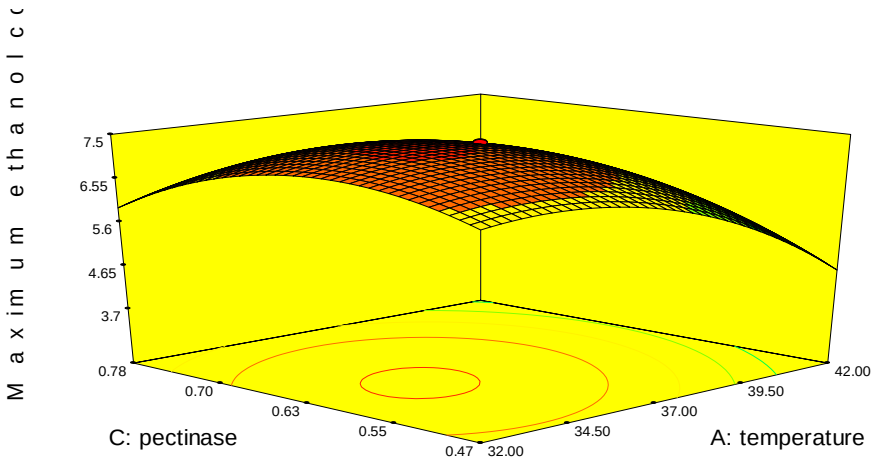
Figure 5.6 (c) illustrates the interactive effect of pectinase and cellulase enzyme on maximum bioethanol concentration at constant incubation temperature (35 °C). Enzyme concentrations play a major role in ethanol production. Loading of the pectinase enzyme increases the bioethanol production, however pectinase loading above ~0.7 mL/g seems to decrease the bioethanol concentration. This confirms earlier conclusions that a high volume of pectinase will degrade polysaccharides.

High concentrations of cellulase (Figure 5.6 (a) and (c)) increase the rate of saccharification, encourages trans-glycosylation reactions, and leads to increased bioethanol concentrations (Vallander and Eriksson, 1985). Cellulase activitiy is inhibited by cellobioase, and to a lesser extent glucose. Several methods have been developed to reduce the inhibition of sugar production during the hydrolysis process, one of which is using high concentrations of enzymes during hydrolysis (Ljunggren, 2005). The optimum loadings for the

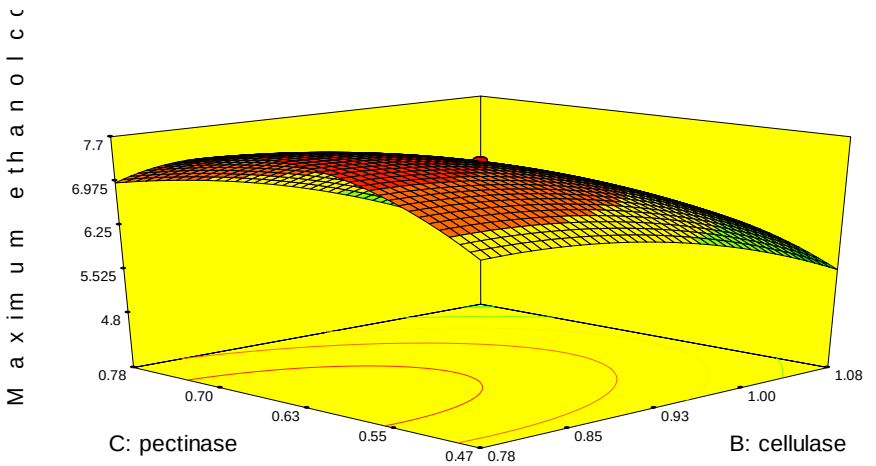
cellulase and pectinase enzymes where found to be, 0.85 mL/g and 0.69 mL/g respectively for the SSF processing of pitaya waste peel.



(a)



(b)



(c)

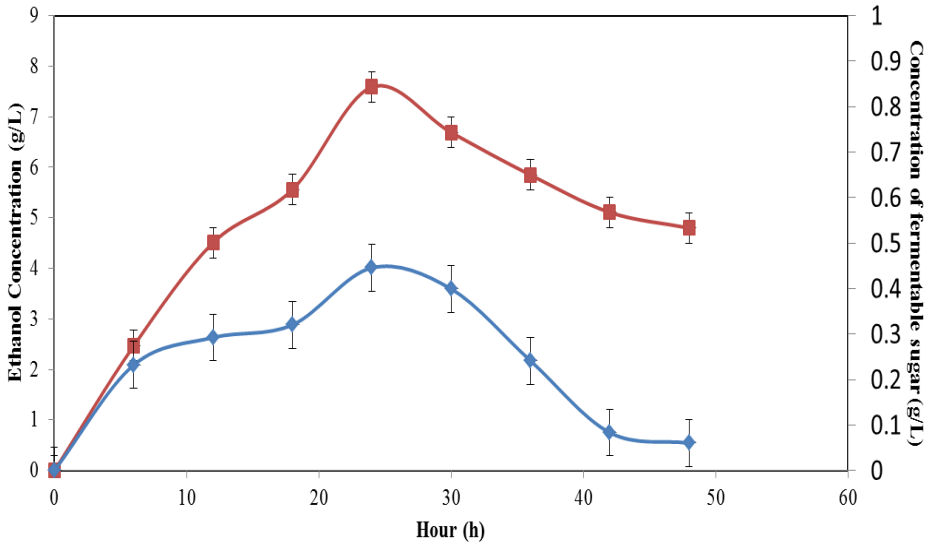
FIGURE 5.6 RESPONSE SURFACE OF MAXIMUM ETHANOL CONCENTRATION

5.3.4 VALIDATION OF OPTIMISED CONDITIONS FOR BIOETHANOL FERMENTATION

The statistically optimized conditions for the SSH process of bioethanol production were temperature, 35.1 °C; cellulase loading, 0.85 mL/g; and pectinase loading, 0.69 mL/g, with a predicted ethanol concentration of 7.57 g/L.

Experiments were carried out to validate the optimal conditions derived from the model equation as shown in Figure 5.7. Under the optimal conditions, the highest level of ethanol achieved was 7.57 g/L. The model predicted that under optimum conditions, the maximum concentration of ethanol would be 7.59 g/L. The maximum fermentable sugar concentration obtained was 0.45 g/L. The desirability function value was determined to be 1.000 for these optimum conditions. The empirical values show that the maximum ethanol obtained from experiment is very close to the value projected by the RSM. The good agreement between the predicted and observed experimental responses serves to verify the accuracy of the mathematical model and the selection of the maximum points.

The standard error was calculated for the validation experiments to be 0.15, indicating that the optimisation process using CCD was suitably applied to the production of bioethanol from pitaya waste peel using Saccharomyces cerevisiae.



Symbols: (■), Ethanol; (♦), Fermentable sugar. Error bars signify the mean ± standard deviation. For data points without error bars, the errors were smaller than the size of the symbol. (Temperature: 35.1 °C, cellulase: 0.85 mL/g, pectinase: 0.69 mL/g).

FIGURE 5.7 ETHANOL PRODUCTION AND FERMENTABLE SUGAR CONCENTRATION UNDER THE STATISTICALLY OPTIMIZED CONDITIONS

5.4 Conclusions

In conclusion, the statistically applied optimization experiments have derived the optimal fermentation conditions for ethanol production in pitaya waste. The substrate loading, initial pH, and inoculums size did not significantly affect ethanol production. The design shows that temperature, cellulase enzyme loading, and pectinase enzyme loading are the factors that affect fermentation in pitaya wastes. These results may assist the understanding of the chief factors and physiological conditions affecting *Saccharomyces cerevisia* cell growth and pitaya waste biosynthesis in future work.

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVE

6.1 Conclusion

This study focused on three main areas of the conversion of pitaya fruit peel waste to ethanol, i.e. the development of a suitable pretreatment strategy to assist the hydrolysis of pitaya waste components into fermentable sugar; the compositional analysis of pitaya waste after pretreatment; and the optimization of the simultaneous saccharification and fermentation (SSF) process for maximum ethanol production.

The chemical composition analysis of pitaya pericarp after pretreatment revealed that the combined pretreatment of hydrothermal and mechanical communition increased the total lignocellulosic component in pitaya pericarp. The highest hemicellulose content (29.28 %) and cellulose content (46.47 %) were recorded in autoclaved, 1 cm cut, pericarp. This study therefore demonstrated that *H.polyrhizus* pericarp can provide cellulosic material suitable for hydrolysis to monomeric sugars.

The combination of coarse mechanical communition and hydrothermal pretreatment presented an optimal cellulosic profile for subsequent fermentation to sugars. The highest sugar concentration of 3.861 g/L after enzymatic hydrolysis came from autoclaved and coarsely cut (1 cm) pericarp, and was therefore

considered the optimal pretreatment for subsequent refinements to the bioethanol production scheme.

The fermentation process using Saccharomyces cerevisiae was done using the simultaneous saccharification and fermentation (SSF) method. A statistical model was developed to determine the maximum bioethanol concentration produced through the batch fermentation process of pitaya pericarp. Response surface methodology (RSM) based on Central Composite Design (CCD) was used to statistically evaluate and optimise the conditions for maximum bioethanol concentration. The parameters chosen in this study were incubation temperature, cellulase enzyme, pectinase enzyme, substrate loading, initial pH, and inoculum size. Using the developed quadratic model equation, a maximum ethanol concentration of 7.57 g/L was achieved in a SSF batch process operating at optimum conditions of incubation temperature (35.1 °C), cellulase enzyme loading (0.85 mL/g), and pectinase enzyme loading (0.69 mL/g).

Mechanically communited and hydrothermally pre-treaded pitaya waste peel is a viable feedstock for bioethanol production.

6.2 Future Perspective

Most cellulosic biomass contains hemicellulose, which can be converted into ethanol to salvage process costs. Simultaneous saccharfication and cofermentation (SSCF) is a near-term processing technology for such conversion. Scale up and reactor design for SSCF could employ the same approach as demonstrated for

SSF in this study. However the kinetic model needs to be extended to accommodate the production and fermentation of pitaya waste sugars. The main factor in setting up a commercial plant is the reduction of capital and operating costs at each stage. This study has extended the understanding of the fundamental processes associated with pretreatment, which can subsequently be used to reduce costs without compromising yields. Future study could focus on enzymes with higher specific activities, to enhance ethanol production and reduce the costs far better than current commercial enzymes. Furthermore, new combinations of organisms could be combined to change different types of sugars into ethanol. Regardless, the new organisms employed must be able to withstand the pressure of a full-scale reactor plant process and toxicity compounds that are present in the sugar hydrolysate.

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