การเพิ่มการผลิตเอทานอลจากกากน้ำตาลโดยการหมักร่วมกับไฮโดรไลเสตของกากมันสำปะหลัง

นางสาวทิพวรรณ วัฒนากรนิยม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

INCREASE OF ETHANOL PRODUCTION FROM MOLASSES BY CO-FERMENTATION WITH CASSAVA WASTE HYDROLYSATE

Miss Thippawan Wattanagonniyom

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Microbiology and Microbial Technology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2015

Thesis Title	INCREASE OF ETHANOL PRODUCTION FROM MOLASSES BY CO-FERMENTATION WITH CASSAVA WASTE HYDROLYSATE
By	Miss Thippawan Wattanagonniyom
Field of Study	Microbiology and Microbial Technology
Thesis Advisor	Associate Professor Ancharida Akaracharanya, Ph.D.

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ทิพวรรณ วัฒนากรนิยม : การเพิ่มการผลิตเอทานอลจากกากน้ำตาลโดยการหมักร่วมกับไฮโดรไลเสตของกากมันสำปะหลัง (INCREASE OF ETHANOL PRODUCTION FROM MOLASSES BY CO-FERMENTATION WITH CASSAVA WASTE HYDROLYSATE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อัญชริดา อัครจรัลญา, 52 หน้า.

การเปรียบเทียบผลการย่อยกากมันสำปะหลัง (ไฮโดรไลเสตของกากมันสำปะหลัง) ด้วยกรดไฮโรคลอริกและเอนไซม์ 3 ชนิดต่อเนื่องกัน (เซลลูเลส, แอลฟา-อะไมเลส และกลูโคอะไมเลส) พบว่าน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยกรดปริมาณ 10% (น้ำหนัก/ปริมาตร) และย่อยด้วยเอนไซม์ปริมาณ 25% (น้ำหนัก/ปริมาตร) มีน้ำตาลรีดิวซ์สูงสุด 0.28 และ 0.14 กรัม/กรัมน้ำหนักแห้ง ตามลำดับ ผลการหมักเอทานอลจากน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยกรดและเอนไซม์โดย *Saccharomyces cerevisiae* TISTR 5606 (SC 90) พบว่าน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยเอนไซม์ให้เอทานอลสูงกว่าน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยกรด 62.47% และเมื่อนำน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยเอนไซม์มาหมักร่วมกับกากน้ำตาลที่ความเข้มข้นน้ำตาลทั้งหมดเริ่มต้น 24.5% (น้ำหนัก/ปริมาตร) พบว่าได้เอทานอลสูงสุด 6.67% (น้ำหนัก/ปริมาตร) และเมื่อเติมโพแทสเซียมไดไฮโดรเจนฟอสเฟต (KH_2PO_4) 0.8% (น้ำหนัก/ปริมาตร) ลงไปหมักร่วมด้วยทำให้เอทานอลที่ได้สูงสุดเพิ่มขึ้นเป็น 7.09% (น้ำหนัก/ปริมาตร) นอกจากนี้การหมักน้ำที่ได้จากการย่อยกากมันสำปะหลังด้วยเอนไซม์ที่มีกากใยมันสำปะหลังที่เหลือหลังการย่อยด้วยเอนไซม์ปริมาณ 3% (น้ำหนัก/ปริมาตร) ผลผลิตเอทานอลสูงสุดที่ได้เพิ่มขึ้นเป็น 7.44% (น้ำหนัก/ปริมาตร)

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5772001523 : MAJOR MICROBIOLOGY AND MICROBIAL TECHNOLOGY
KEYWORDS: MOLASSES / CASSAVA WASTE PULP / HYDROLYSATE / ETHANOL / S. CEREVISIAE

THIPPAWAN WATTANAGONNIYOM: INCREASE OF ETHANOL PRODUCTION FROM MOLASSES BY CO-FERMENTATION WITH CASSAVA WASTE HYDROLYSATE. ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, Ph.D., 52 pp.

Comparison of cassava waste pulp hydrolysate obtained from hydrochloric acid and 3 different enzymes (cellulase, alpha-amylase and glucoamylase). Consequently hydrolysis, revealed that 10% and 25% (w/v) substrate loading of The CWP-acid and enzymatic hydrolysate contained maximal reducing sugar at 0.28 and 0.14 g/g dry weight, respectively. But the CWP-enzymatic hydrolysate gave 62.47% higher ethanol than the CWP-acid hydrolysate when they fermented by *Saccharomyces cerevisiae* TISTR 5606 (SC 90). Co-fermentation of molasses with the CWP-enzymatic hydrolysate at initial total sugar 24.5% (w/v) yielded maximal ethanol 6.67% (w/v). Fermentation of the molasses-CWP enzymatic hydrolysate mixture by supplementation with 0.8% (w/v) KH_2PO_4 increased maximal ethanol to 7.09% (w/v). This maximal level of ethanol could be increased to 7.44% (w/v) by addition of 3% (w/v) of solid residue obtained after enzymatic hydrolysis of cassava waste pulp.

Department:	Microbiology	Student's Signature	.....
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	.....
Academic Year:	2015		

ACKNOWLEDGEMENTS

This research would not success without some people or some budgets to support me and my research.

Firstly, I would like to express my sincere gratitude to my advisor Associate Professor Dr. Ancharida Akaracharanya for her advice, suggestion, kindliness, love and care in all of my research, study and life.

Beside my advisor, I would like to thank my thesis committee: Assistant Professor Dr. Suchada Chanprateep Naprathorn, Associate Professor Dr. Suthep Thaniyavarn, Associate Professor Jiraporn Thaniyavarn and Associate Professor Dr. Vichien Kitpreechavanich, for their checking, comment, opinion and guidance my thesis.

I am grateful to everybody in Department of Microbiology especially member of Associate Professor Dr. Ancharida Akaracharanya laboratory for give me the knowledge, helping, support, friendship and fortitude.

I am thankful to the scholarship from the Graduate school, Chulalongkorn University to commemorate the 72nd Birthday Anniversary of His Majesty the King Bhumibol Aduladej is gratefully acknowledged, for my study budget and Government budget, Fiscal year 2015 for my research budget.

My sincere thanks also goes to my family for the continuous support, love and always stay beside me.

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Introduction

1.1 Background

Our world had a main energy was fossil fuel but the problem of depletion and environmental pollution caused of research and development of alternative energy. Bioethanol is one of alternative energy that produce by microorganism. The 1st generation of bioethanol produce from agricultural product such as sugarcane, corn and cassava. As it is food, it cause limited feedstock then next generation of ethanol production was produced from agroindustrial waste such as molasses and cassava waste pulp, agricultural waste such as rice straw and corn stover and other lignocellulosic biomass. (Naik, Goud, Rout, & Dalai, 2010)

Molasses is a waste from sugar factory. It is a famous substrate for ethanol fermentation because it was easily used by no need pretreatment or hydrolysis process (Hatano et al., 2009) and also contain high sucrose and nutrient which important for growth and fermentation. However, addition of some nutrients can increase efficiency of ethanol production from molasses such as magnesium sulfate and manganese(II) chloride could increase activity of invertase enzyme in ethanol process of yeast strain X2180-1B (Takeshige & Ouchi, 1995) and urea, magnesium sulfate and soybean flour also could increase ethanol production from molasses by *Saccharomyces bayanus* because urea and soybean flour are nitrogen source for yeast and magnesium ion is coenzyme in ethanol fermentation pathway (Pradeep & Reddy, 2010).

Cassava waste pulp (CWP) is waste from cassava starch industry that have waste volume in Thailand around 7 million tons per year (Deeprasert, Boonmunma, Boottongmol, Chinchoedwong, & Loha, 2011). Its compositions are 50.0% (w/w) starch, 5.3% (w/w) protein and 35.9% (w/w) lignocellulosic fiber that can use as mushroom cultivation material, fertilizer or animal feed. Due to low protein content, it is not used as feed. So, cassava waste pulp is still remain and emiss unpleasant odor from fermentation of microorganism. In 2010, Thongchul et al. reported using of

cassava waste pulp as substrates for lactic and ethanol production by *Rhizopus oryzae* and found acid hydrolysate gave the higher reducing sugar than enzymatic hydrolysate. Both acid and enzymatic hydrolysate could be use as substrate for the ethanol production with out difference in ethanol yield but the maximum glucose concentration liberated from cassava waste pulp hydrolysis has only 10% (w/v) that is not break even for ethanol production which usually use around 16-24% (w/v). However, their hydrolysate also contains some nutrient for yeast and low nitrogen composition that encourage ethanol production more than growth (Danbamrongtrakool, Tungcharoen, & Parakulsuksatid, 2014).

Therefore, we interested in using cassava waste pulp as supplement in ethanolic molasses fermentation. This partial replacement of molasses with cassava waste pulp reduces production cost and also reduces molasses demand.

1.2 Objectives

1. Comparison of CWP-acid hydrolysate and CWP- enzymatic hydrolysate on ethanol production by *Saccharomyces cerevisiae* TISTR 5606 (SC 90).
2. Optimization of ethanol production from molasses-CWP hydrolysate mixture.

CHAPTER II

Literature review

2.1 Molasses

Molasses (Figure 1) is by product from sugar production which start from extraction of sugarcane by crusher rollers then separate baggage out from juice. The juice was clearified by add calcium oxide (lime) and heat for precipitate insoluble substance. Clearified juice was concentrated by vacuum evaporation for remove water around 70% out. At this point, clearified juice was changed to syrup that will be continue to crystallization at vacuum pan. The syrup was evaporated until saturated point and get dense mixture of syrup and sugar crystals, called massecuite. Massecuite was centrifuged to separate sugar crystals (Figure 2). The waste from centrifugation is the molasses that is viscous syrup and dark brown color.

Figure 1 *Molasses*
(Patil, Premalatha, Rao, & Ganavi, 2013)

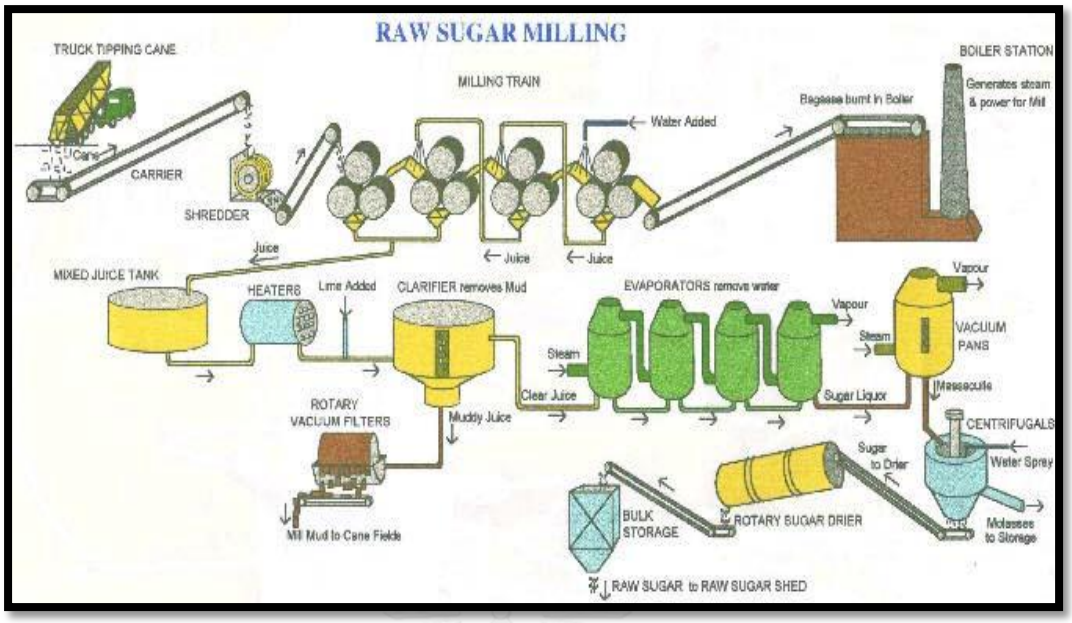
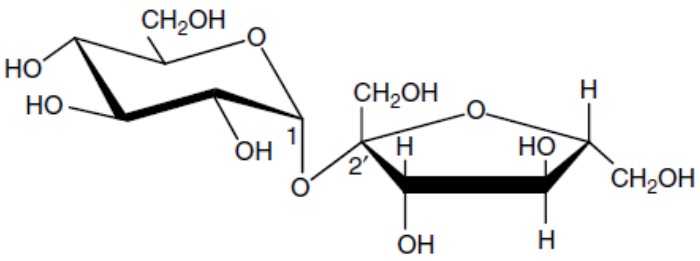


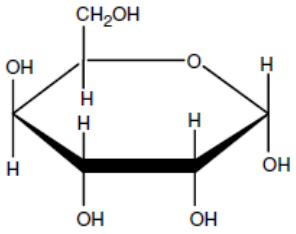
Figure 2 *Sugar production process*
(NSW Sugar Milling Co-operative, 2006 : online)

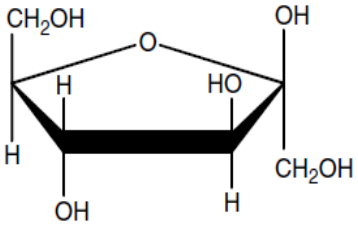
2.2 Molasses as substrate for ethanol production

Molasses is a popular substrate in ethanol industry because it is a cheap substrate, not require pretreatment process and has high fermentable sugar content is sucrose 30-40% (w/w), glucose 4-9% (w/w) and fructose 5-12% (w/w) (Figure 3). In addition, it also contains protein, vitamin (riboflavin, thiamine and biotin) and minerals (K, Ca, Na, Mg, Cu, Fe, Mn, Zn, Cl and S) which essential for microbial fermentation (Chotineeranat et al., 2010). Although, molasses has a lot of nutrient, it can increse ethanol production by addition of other nutrients such as urea, $MgSO_4$ and soybean flour (Pradeep & Reddy, 2010).



A.





B. C.

Figure 3 *Sugar structure (A.) Sucrose (B.) Glucose (C.) Fructose* (Cui, 2005)

2.3 Cassava waste pulp

Cassava is a third major crop product in Thailand. It has cultivation area around 1 million hectares that can produce cassava root about 16 million tons per year (Office of the national economic and social development board, 2006). 60% of cassava root was used in cassava starch industry by start from wash cassava root then cut and rasp to small piece and extract starch (Figure 4). Cassava waste pulp was remained after extraction step around 83% which generated greater than 7 million tons per year. This waste can use for feed or fertilizer but it can not use all waste because low quality of cassava waste pulp which contain high of starch (50.0%(w/w dry pulp)) and lignocellulose (35.9%(w/w dry pulp)) but low protein content (5.3%(w/w dry pulp)) (Thongchul, Navankasattusas, & Yang, 2010). Its microbial fermentation causes unpleasant odor emission.

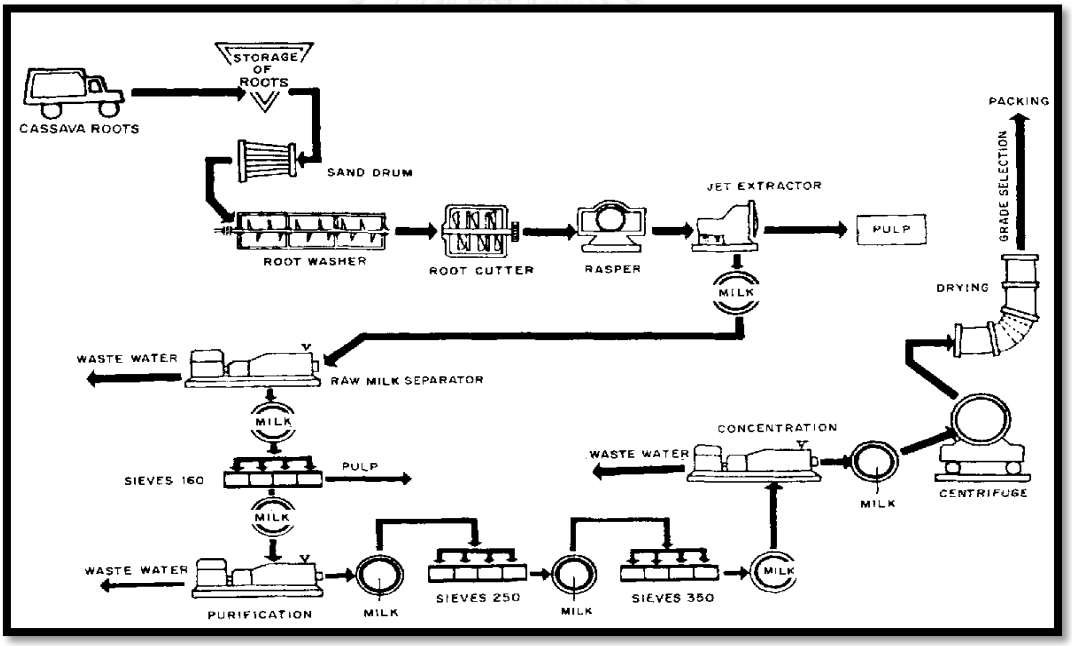


Figure 4 *Cassava starch production process*
(Agriculture and Consumer Protection, 2016 : online)

2.4 Cassava waste pulp as substrate for ethanol production

From the major composition of cassava waste pulp, starch and lignocellulose, it can use as substrate for ethanol production after hydrolysis.

2.4.1 Starch (Sriroth & Piyachomkwan, 2007)

2.4.1.1 Starch composition and structure

Starch ($(C_6H_{10}O_5)_n$) is carbohydrate consist of a lot of glucose that have two structures are 15-20% linear amylose and 80-85% branched amylopectin. Amylose was joined by α1→4glycosidic bonds (Figure 5) and amylopectin joined by α1→4glycosidic bonds in linear section and α1→6 glycosidic bonds in branch section (Figure 6).

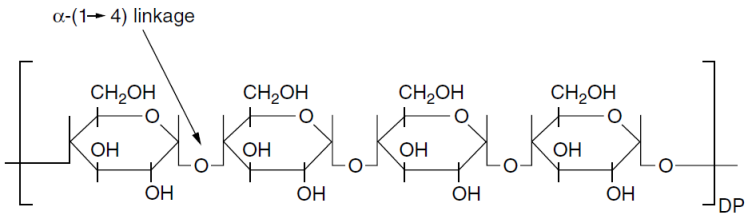


Figure 5 *Structure and linkage of amylose*
(Cui, 2005)

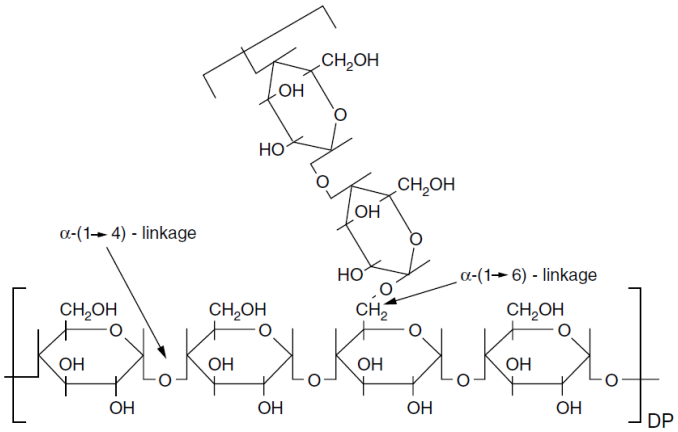


Figure 6 *Structure and linkage of amylopectin*

(Cui, 2005)

2.4.1.2 Starch hydrolysis

It can change to monosaccharide by two methods are acid and enzymatic hydrolysis.

2.4.1.2.1 Acid hydrolysis

Acid such as HCl, H_2CO_3, H_2SO_4 and HNO_3 can hydrolyse starch by H^+ ion release from acid in high temperature condition and break glycosidic bound. Efficiency of hydrolysis is up to acid concentratration, type of acid, time and temperature. Suitable condition will be give high released sugar and low inhibitor.

2.4.1.2.2 Enzymatic hydrolysis

Starch enzymatic hydrolysis has two steps are liquefaction and saccharification

2.4.1.2.2.1 Liquefaction

Starch was hydrolysed to gelatinization at 60- 70°C and was hydrolysed α1→4glycosidic bonds of amylose and amylopectin by alpha- amylase enzyme at 100- 105°C. Viscosity of gelatinization was decreased. Maltodextrin and polysaccharide are products of this step.

2.4.1.2.2.2 Saccharification

Glucoamylase hydrolyses α1→4 and α1→6 glycosidic bonds of maltodextrin and polysaccharide to glucose at 60°C. Glucose in this step is used as substrate.

2.4.2 Lignocellulose

2.4.2.1 Lignocellulose composition and structure (Punnapayak & Prasongsuk, 2015)

Lignocellulose, major component of cell wall, which is the most abundant raw material on the world. Its structure composed of cellulose, hemicellulose and lignin which is very strong structure (Figure 7).

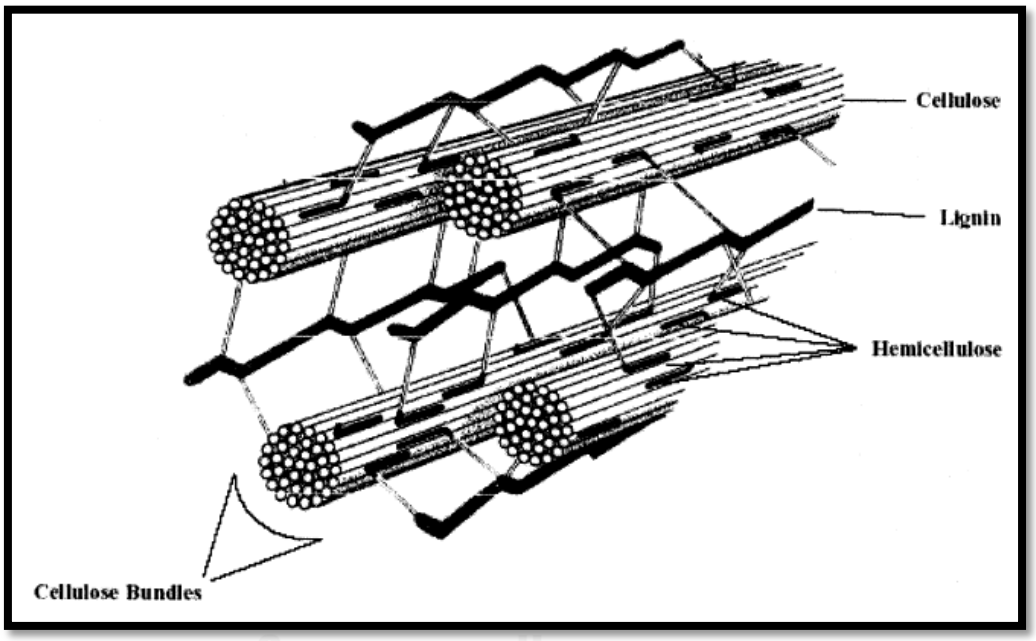


Figure 7 *Lignocellulose composition*
(Gnansounou & Dauriat, 2005)

2.4.2.1.1 Cellulose

Cellulose (30–60%, w/w dry weight) consisted of unbranched glucose polymer was linked by β1→4glycosidic bonds (Figure 8). Besides, It has vander waals forces and hydrogen bound link between each glucan in crystalline structure. Hence, it is strong and difficult to disruption.

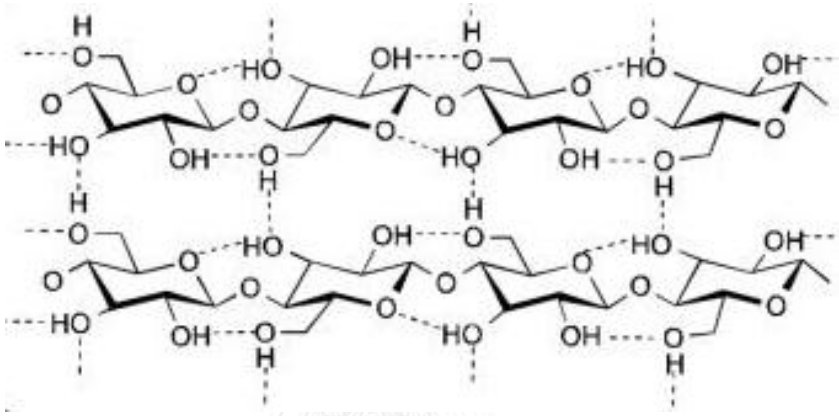


Figure 8 *Cellulose structure*
(Fu et al., 2015)

2.4.2.1.2 Hemicellulose

Hemicellulose (20–40%, w/w dry weight) presents along with cellulose in cell wall. It can not dissolve in water but it easy dissolve in strong base. Hemicellulose has major sugar as hexose (Carbon 6 atom) ; β-D-galactopyranose, β-D-glucopyranose and β-D-mannopyranose; and pentose (Carbon 5 atom), α-L-arabinopyranose, β-D-xylopyranose and α-L-arabinofuranose. Its backbone sugar is linked by 1→4 bound (Figure 9).

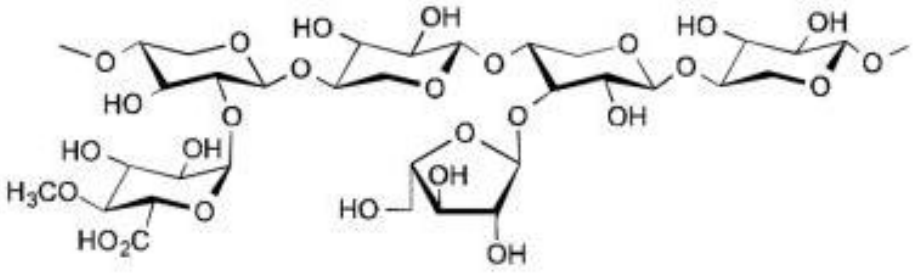


Figure 9 *Hemicellulose structure*
(Fu et al., 2015)

2.4.2.1.3 Lignin

Lignin (15–25%, w/w dry weight) is outer component of lignocellulose. It makes fiber strong and protects cell wall from chemical and biological degradation because it is hydrophobic and complex structure of phenyl propane unit (Figure 10).

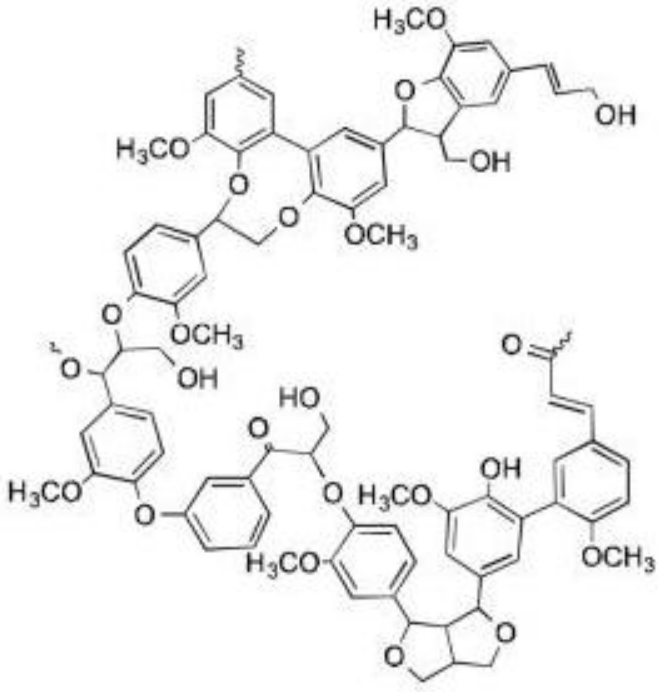


Figure 10 *Lignin structure*
(Fu et al., 2015)

2.4.2.2 Lignocellulose hydrolysis (Ruangviriyachai & Niwaswong, 2012)

Lignocellulose can be easily hydrolysed to fermentable sugar after pretreatment.

2.4.2.2.1 Pretreatment

Pretreatment is the first step to increase efficiency of hydrolysis because it breaks complex structure or remove lignin from hemicellulose and cellulose that obstruct hydrolysis (Figure 11). There are 4 pretreatment methods.

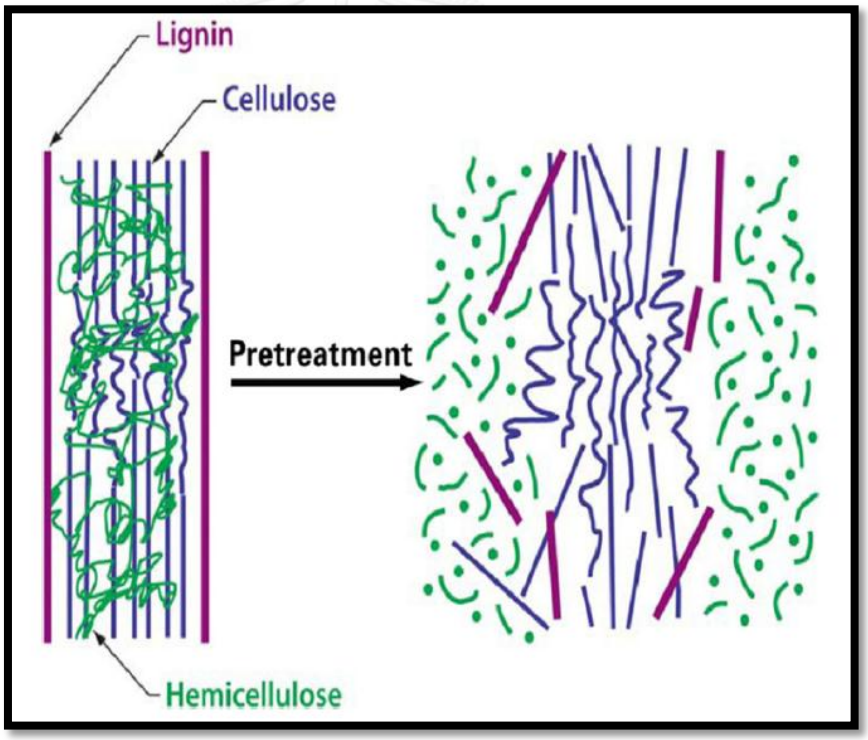


Figure 11 *Pretreatment of biomass*
(Bhatia, Johri, & Ahmad, 2012)

2.4.2.2.1.1 Physical pretreatment

This method breakdowns the structure by grinding or cutting. It reduces lignocellulose size and increases surface area of reaction.

2.4.2.2.1.2 Chemical pretreatment

This method breakdowns the structure by using acid or base to remove lignin and hemicellulose.

2.4.2.2.1.3 Physico – chemical pretreatment

This method breakdowns the structure by using mixture of physical and chemical method such as dilute base in high temperature and pressure condition.

2.4.2.2.1.4 Biological pretreatment

This method breakdowns the structure by using enzyme decrease lignocellulose crystal. This pretreatment is suitable for enzymatic hydrolysis.

2.4.2.2.2 Hydrolysis

Hydrolysis is process which reduce polysaccharide size to five and six carbon atoms of sugar such as xylose, mannose, arabinose and glucose and cellulose. These monomer sugar can use as substrate for microorganism.

2.4.2.2.2.1 Acid hydrolysis

Polysaccharide was hydrolysed by acid solution in high temperature and pressure condition. Hydrolysate from this hydrolysis must do neutralization before fermentation because pH value not suitable for microorganism. This hydrolysis has advantage is fast and convenient but it also has disadvantage is formation of inhibitor compound such as 5-hydroxy-

methylfurfural (HMF) (Inhibitor from cellulose), Furfural (Inhibitor from hemicellulose) and Vanillin (Inhibitor from lignin) (Hatano, Aoyagi, Miyakawa, Tanokura, & Kubota, 2013) (Figure 12). These inhibitor can effect to activity of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and pyruvate dehydrogenase (PDH) in growth and ethanol fermentation pathway (Modig, Lidén, & Taherzadeh, 2002) (Figure 13).

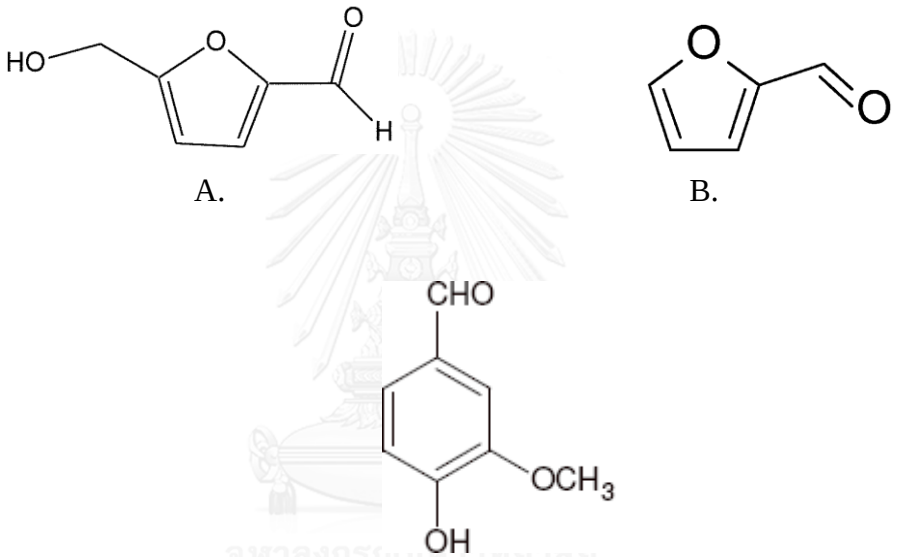


Figure 12 *Chemical structure of (A.) 5-hydroxy-methylfurfural (HMF), (B.) Furfural and (C.) Vanillin*
((A.) (Subramaniam, Zuo, Busch, & Venkitasubramanian, 2012)
(B.) (Danon, Marcotullio, & de Jong, 2014) and (C.) (Converti, Aliakbarian, Domínguez, Vázquez, & Perego, 2010)

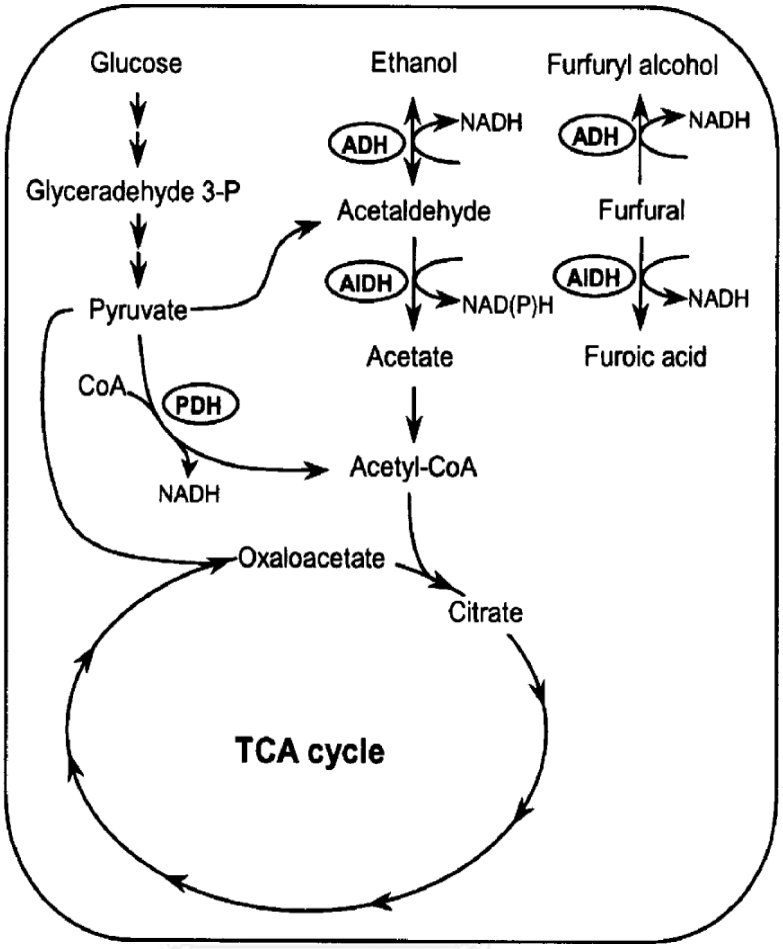


Figure 13 *Alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and pyruvate dehydrogenase (PDH) in growth and ethanol fermentation pathway of yeast* (Modig et al., 2002)

2.4.2.2.2.2 Enzymatic hydrolysis

Cellulose was hydrolysed by cellulase enzyme consist of endoglucanase, exoglucanase (CBH) and β-glucosidase. Amorphous cellulose was random hydrolysed by endoglucanase then exoglucanase hydrolyse end of amorphous or crystalline cellulose to oligomers or cellobiose and β-glucosidase simultaneously hydrolyse cellobiose to glucose. (Goyal, Ghosh, & Eveleigh, 1991) (Figure 14)

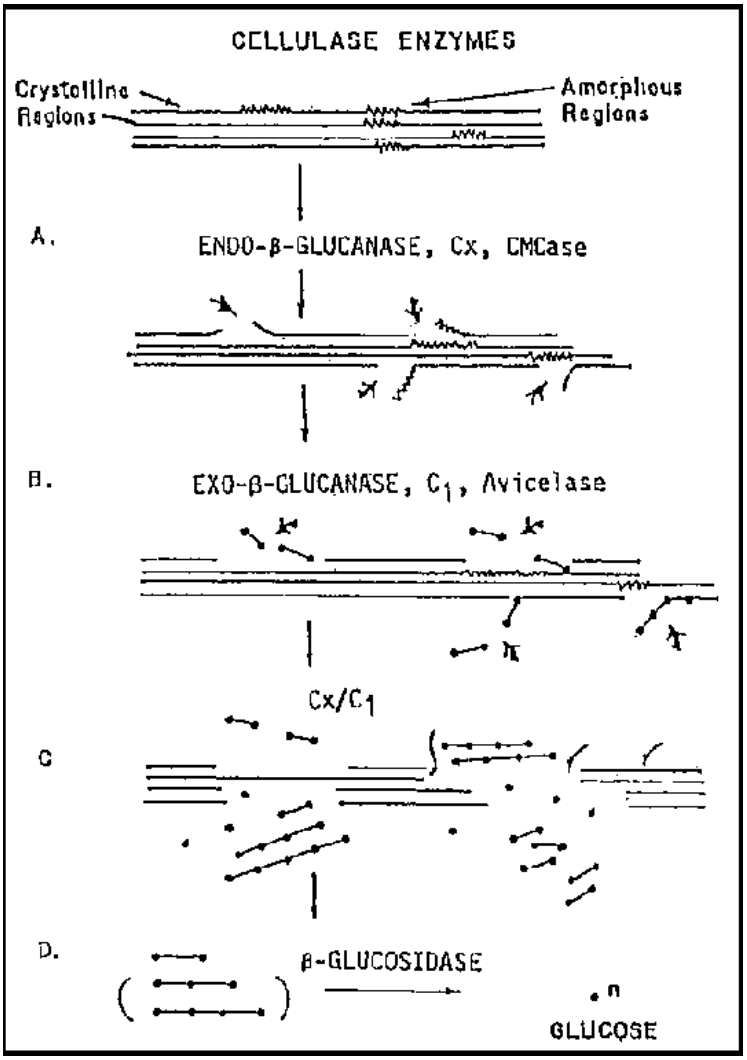


Figure 14 *Action of cellulase enzyme*

(Agriculture and Consumer Protection, 2016 : online)

2.5 Ethanol production

Ethanol can produce from both of chemical and biological methods.

2.5.1 Hydration of ethylene

This is chemical production which produce from ethylene by hydration (Figure 15).

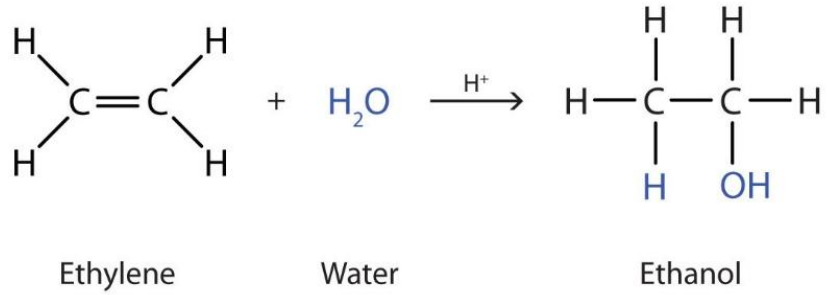


Figure 15 *Hydration of ethylene*
(Ball, Hill, & Scott, 2016 : online)

2.5.2 Fermentation

This is biological production that produce from fermentable sugar of agricultural product or agricultural waste by yeast or some bacteria. These microorganism transfer fermentable sugar to glycolysis pathway (Embden-Meyerhof-Parnas pathway) for converse to pyruvate and metabolize to ethanol in anaerobic condition by alcohol dehydrogenase and aldehyde dehydrogenase enzyme. (Punnapayak & Prasongsuk, 2015) (Figure 13)

2.6 Microorganism for ethanol production

Ethanol can produce from microorganism that can converse fermentable sugar in substrate to ethanol such as bacteria, fungi and yeast. Ng et al. (1981) reported that co-culture of bacteria was *Clostridium thermohydrosulfuricum* and *C. thermocellum* could produce high ethanol and acetate higher than monoculture. Fujio et al. (1985) studied ethanol production from raw cassava starch by fungi was *Rhizopus koji* in a gas circulation type fermentor and found *Rhizopus koji* could produce ethanol yield reached 83.5-72.3% of the theoretical yield and Najafpour et al. (2004) reported *Saccharomyces cerevisiae* (yeast) in an immobilized cell reactor could produce ethanol higher than batch fermentation was 33.6%. However, yeast especially *Saccharomyces cerevisiae* (Figure 16) is a famous microorganism which was used in ethanol industry.

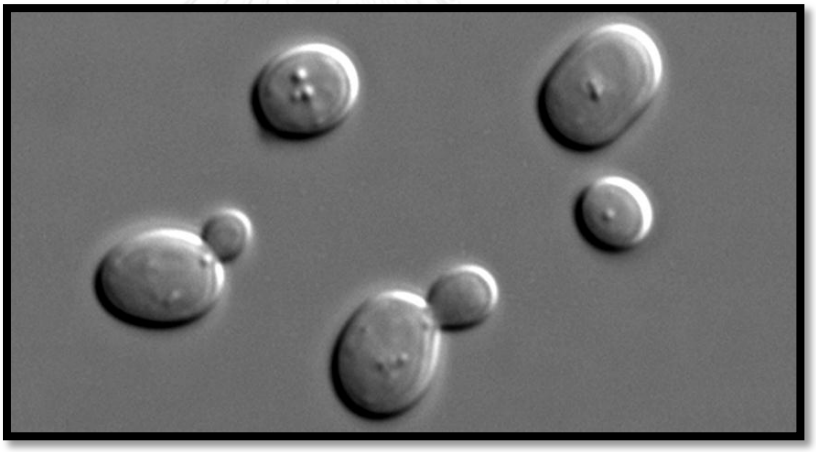


Figure 16 *Saccharomyces cerevisiae under a microscope*

(Saey, 2016 : online)

2.7 Affecting condition of ethanol fermentation

2.7.1 Temperature

Suitable temperature greatly lead to desirable growth and ethanol production because temperature affect to enzyme activity, membrane turgidity, protein denaturation, protein synthesis and membrane fluidity of yeast cell. (Limtong, 1997; Tesfaw & Assefa, 2014)

2.7.2 pH

pH of medium affect to enzyme activity in growth and ethanol production. Moreover, It can control contaminated microbe that disturb process and product. pH adjustment is up to buffer capability of fermentation medium. If medium has low buffer capability, pH was adjusted to 5.5. On the other hand, pH was adjusted to 4.5-4.7 in high buffer capability. (Limtong, 1997)

2.7.3 Initial sugar concentration

Sugar is substrate of yeast for growth and ethanol fermentation. High sugar concentration has advantage was inhibit undesirable microbe and give high ethanol production but it also has disadvantage from over concentration that affect to yeast cell. Over concentration has osmotic pressture which force cell plasmolysis and enzyme activity was inhibited (Limtong, 1997). *Saccharomyces cerevisiae* was studied effect of glucose concentrations on ethanol production and found ethanol production in YPD medium (various glucose concentrations at 74, 120, 170, 220, and 270 g/L) at 220 g/l glucose concentration gave highest ethanol production was 85.87 g/L at 40°C, 150 rpm agitation (Wilaithup, Laemsak, Sirisansaneeyakul, Vanichsriratana, & Parakulsuksatid, 2013).

2.7.4 Supplement

Supplement can enhance growth and ethanol production but it should has suitable concentration. Yeast extract and $(NH_4)_2SO_4$ are nitrogen source of yeast which must has suitable concentration in fermentation medium because over nitrogen concentration lead to growth more than ethanol fermentation (Thongchul et al., 2010). Moreover, yeast need many nutrient for increase activity such as Potassium (Na^+/K^+ ATP pump), Magnesium (increase Hexokinase & Invertase activity, against ethanol stress and increase sugar conversion), cupper (coenzyme of cytochrome C oxidase in oxidative phosphorylation), calcium (against ethanol stress and increase sugar conversion), zinc (coenzyme of alcohol dehydrogenase and increase sugar conversion) and manganese (increase pyruvate carboxylase and Invertase activity) (Pereira, Guimarães, Teixeira, & Domingues, 2010).

2.7.5 Ethanol concentration

High ethanol concentration in fermentation broth affect to yeast cell because ethanol can inhibit RNA and protein synthesis, denature protein, decrease efficacy of cell membrane (lipid aggregate) and decrease dehydrogenase and hexokinase activity (Limtong, 1997). Therefore, fermentation medium was adjusted initial sugar concentration for suitable ethanol production or yeast was increased ethanol tolerance limit by strain improvement such as expression of TPS1 Gene in Saccharomyces sp. W0 enhances trehalose accumulation. Trehalose is Non-reducing disaccharide that can protects cells against various stress conditions (Cao, Chi, Liu, & Chi, 2014).

2.7.6 Carbon dioxide concentration

Carbon dioxide effect to yeast cell in both of anaerobic and aerobic condition. It change capability of cell membrane and pH of fermentation medium (carbon dioxide dissolve in the fermentation medium). However, Its stress can reduce by relief in saturated copper sulfate (antibiotic) (Limtong, 1997).

2.8 Improvement of ethanol production by Immobilization

Immobilization is a improvement method of ethanol production because it increase surface area for cell adsorption and can protect cell from stress such as sugar concentration, ethanol concentration, carbon dioxide concentration, inhibitor and shearing force (Tesfaw & Assefa, 2014). Immobilizing substrate can be divided into two types are synthetic substrate such as photocrosslinkable resin beads which was reported that ethanol production of immobilized *Saccharomyces cerevisiae* cells on photocrosslinkable resin beads give higher than free cell in repreated batch fermentation (120 hours, 5 batches) (Watanabe et al., 2012) and natural substrate such as palm pressed fiber (Figure 17) which was reported that ethanol production of immobilized *Candida shehatae* TISTR5843 on palm press fiber can produce ethanol higher than free cell in both of batch and repreat batch (96 hours, 4 batches) (Riansa-ngawong, Suwansaard, & Prasertsan, 2012).

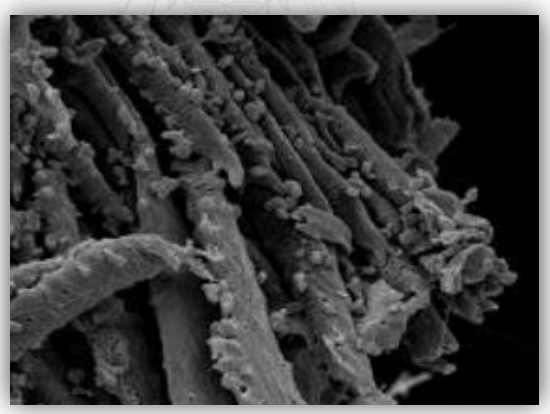


Figure 17 *Immobilized cells on delignified small palm press fiber (1500 X)*
(Riansa-ngawong et al., 2012)

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microorganism

Saccharomyces cerevisiae TISTR 5606 (SC 90) was received from Thailand Institute of Science and Technology

3.1.2 Casava waste pulp (CWP)

Cassava waste pulp (CWP) collected from Sa-nguan Wong Industry Co., Ltd., Nakhon Rachasima province, Thailand was kept at –20°C and thawn at room temperature before use.

3.1.3 Molasses

Molasses was collected from Khonburi Sugar Co., Ltd., Nakhon Rachasima province, Thailand. It was kept at 4°C until use.

3.1.4 Enzymes

Cellulase (Accellerase^{TM}1500; 2500 CMC U/g and 650 pNPG U/g), Alpha-amylase (Spezyme alpha; 13,775 AAU/g) and glucoamylase (GC 147; 580 TGAU/g) (Genencor, Danisco US Inc., USA.) were a gift from Thai Alcohol Co., Ltd., Nakhonpathom province, Thailand.

3.1.5 Equipment

3.1.5.1 -20°C Freezer: model MDF-U536D, Sanyo Electric Biomedical Co., Ltd., Japan.

3.1.5.2 -80°C Freezer: model MDF-U71V, Sanyo Electric Biomedical Co., Ltd., Japan.

3.1.5.3 Autoclaves: model SS-325, Tomy and model HV-28, Hirayama Manufacturing Corp., Japan.

3.1.5.4 Fermentor: model MDL-8C, B.E., Marubishi Co., Ltd., Japan.

3.1.5.5 Gas chromatography (GC): model GC 2010, Shimadzu Corp., Japan.

3.1.5.6 Hot air oven: model UE 600, Memmert GmbH + Co.KG, Germany.

3.1.5.7 High speed centrifuge: model 1920, Kubota Corp., Japan.

3.1.5.8 Incubator: model INE500, Memmert GmbH + Co.KG, Germany.

3.1.5.9 Incubator shaker: model Innova 4330, New Brunswick Scientific Co., Inc., U.S.A.

3.1.5.10 Laminar flow: model V6, Lab service Ltd., Thailand.

3.1.5.11 Magnetic stirrer: Model 502P-2, Thermolyne Corp., U.S.A.

3.1.5.12 Micropipette, Eppendrof (Thailand) Co., Ltd., Thailand.

3.1.5.13 Microscope: model CH30RF200, Olympus Corp., Japan.

3.1.5.14 Microwave: model R-311, Sharp Corp., Japan.

3.1.5.15 pH meter: model Seven Easy, Mettler Toledo Ltd., Switzerland.

3.1.5.16 Precision balance: model PG6002-S and AG285, Mettler Toledo Ltd., Switzerland.

3.1.5.17 Refrigerated centrifuge: Allegra 25R, Beckman coulter, Inc, USA.

3.1.5.18 Spectrophotometer: model Genesys 10S UV-Vis, Thermo Fisher Scientific Inc., USA.

3.1.5.19 Vortex mixer: model G560, Scientific Industries, Inc., USA.

3.1.5.20 Water bath shaker: model Gyromax 939XL, Amerex Instrument, Inc., USA and model SS40-D, Grant Instrument Ltd., UK.

3.1.6 Chemicals (Analytical grade)

3.1.6.1 Agar, Becton, Dickinson and Company, USA.

3.1.6.2 Ammonuimmolybda tetetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$), Merck Co., Ltd., Germany.

3.1.6.3 Ammonium sulphate ($(NH_4)_2SO_4$), Merck Co., Ltd., Germany.

3.1.6.4 Bacto-peptone, Becton, Dickinson and Company, USA.

3.1.6.5 Copper (II) sulfate pentahydrate ($CuSO_4\cdot 5H_2O$), Merck Co., Ltd., Germany.

3.1.6.6 di-Sodium arsenate heptahydrate ($Na_2HAsO_4\cdot 7H_2O$), Sigma-Aldrich Inc., USA.

3.1.6.7 di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4\cdot 12H_2O$), Merck Co., Ltd., Germany.

3.1.6.8 Glucose ($C_6H_{12}O_6$), Sigma-Aldrich Inc., USA.

3.1.6.9 Glycerol ($C_3H_8O_3$), Merck Co., Ltd., Germany.

3.1.6.10 Hydrochloric acid (HCl), Sigma-Aldrich Inc., USA.

3.1.6.11 Magnesium sulphate heptahydrate ($MgSO_4\cdot 7H_2O$), Sigma-Aldrich Inc., USA.

3.1.6.12 Phenol (C_6H_5OH), Merck Co., Ltd., Germany

3.1.6.13 Potassium dihydrogen phosphate (KH_2PO_4), Merck Co., Ltd., Germany.

3.1.6.14 Potassium sodium tartrate pentahydrate ($KNaC_4H_4O_6\cdot 5H_2O$), Merck Co., Ltd., Germany.

3.1.6.15 Sodium hydroxide (NaOH), Merck Co., Ltd., Germany.

3.1.6.16 Sodium sulphate (Na_2SO_4), Merck Co., Ltd., Germany.

3.1.6.17 Sucrose ($C_{12}H_{22}O_{11}$), Merck Co., Ltd., Germany.

3.1.6.18 Sulfuric acid (H_2SO_4), Merck Co., Ltd., Germany.

3.1.6.19 Yeast extract, Becton, Dickinson and Company, USA.

3.2 METHODS

3.2.1 Various substrate loading of cassava waste pulp hydrolysis

3.2.1.1 Acid hydrolysis (Thongchul et al., 2010)

Cassava waste pulp in HCl solution (1g dry weight / 0.33g HCl) was hydrolysed at 121°C for 15 min then filtered to remove solid residue. CWP-acid hydrolysate (filtrate) was analysed for residual sugar by Somogyi-Nelson method (Somogyi, 1952) for comparison of reducing sugar released from various substrate loading level (10, 20 and 30%, w/v).

3.2.1.2 Enzymatic hydrolysis (Thongchul et al., 2010)

Cassava waste pulp suspended in deionized water (1 g dry weight / 4 ml deionized water) was autoclaved at 121°C for 15 min then hydrolysed by cellulase (1.41 CMC U/g) at 50°C for 24 h, α-amylase (48 U/g) at 85°C for 1 h and glucoamylase (4.8 U/g) at 60°C for 3 h. Resultant cassava waste pulp slurry (CWP-enzymatic hydrolysate containing solid residue of CWP) was filtered and CWP-enzymatic hydrolysate was analysed for residual sugar. CWP-enzymatic hydrolysate at 25 and 50% w/v substrate loading and reducing sugar released was compared.

3.2.2 Comparison of ethanol production from CWP-acid and enzyme hydrolysate

3.2.2.1 Inoculum preparation

Single colony of *Saccharomyces cerevisiae* TISTR 5606 (SC 90) grown on YPD agar at 30°C for 48 h was inoculated into YPD broth and incubated at 30°C, 200 rpm for 24 h. The culture was transferred at 1% (v/v) to the same medium and incubated at the same condition for 18 h (late log phase culture). Number of cell in the obtained culture was determined by haemacytometer then centrifuged at 4°C, 8000 rpm for 15 min to precipitate cells. Resultant cell pellet was used as inoculum.

3.2.2.2 Fermentation medium preparation

The pH of CWP-acid or CWP-enzymatic hydrolysate was adjusted to 5.5 after adding 0.2% (w/v) $(NH_4)_2SO_4$ then steriled by autoclaving at 110°C for 10 min

3.2.2.3 Ethanol fermentation

The inoculum was inoculated into fermentation medium (final 10^8 cells/ml) and incubated at 30°C, 100 rpm mixing, for 48 h under oxygen limit condition.The oxygen limit condition was performed by capping the flask with rubber stopper connected to air – lock containing saturated copper sulfate solution. The ethanol produced in cell-free culture was analysed by Gas chromatography (GC) after centrifugation at 4°C, 13,000 rpm for 10 min.

3.2.3 Effect of nutrients supplementation in molasses–CWP enzymatic hydrolysate on ethanol production

Twenty six point one grams (26.10g) molasses containing 23.0% (w/v) total sugar was dissolved in 100 ml of CWP-enzymatic hydrolysate containing 3.5% (w/v) total sugar. Then the molasses–CWP enzymatic hydrolysate was supplemented with nutrients, (0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.075% $MgSO_4.7H_2O$ and 1% yeast extract (w/v)) as shown in Table 1 adjusted pH to 5.5 and autoclaved at 110°C for 10 min. The supplemented molasses–CWP enzymatic hydrolysate was fermented to ethanol and analysed for ethanol produced as described in 3.7.3.

Table 1 Nutrients supplementation in molasses–CWP enzymatic hydrolysate

Supplements (w/v)	Medium No.															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0.2% $(NH_4)_2SO_4$	+	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-
0.2% KH_2PO_4	+	+	-	+	+	+	-	-	+	+	-	-	-	-	+	-
0.075% $MgSO_4 \cdot H_2O$	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-	-
1% Yeast extract	+	+	+	+	-	-	-	+	-	+	+	+	-	-	-	-

3.2.4 Effect of KH_2PO_4concentration in molasses–CWP enzymatic hydrolysate on ethanol production

Molasses–CWP enzymatic hydrolysate was supplemented with various concentration of KH_2PO_4 (0, 0.2, 0.4, 0.6, 0.8 and 1%, w/v) and fermented to ethanol as described in 3.7.3.

3.2.5 Effect of initial total sugar concentration in molasses–CWP enzymatic hydrolysate on ethanol production

Molasses-CWP enzymatic hydrolysate at various initial total sugar (20.5, 22.5, 24.5 and 26.5%, w/v) was supplemented with 0.8% (w/v) KH_2PO_4, steriled at 110°C for 10 min then fermented to ethanol. Centrifugation was performed to separate for supernatant and analysed for ethanol produced.

3.2.6 Effect of CWP solid residue in molasses–CWP enzymatic hydrolysate on ethanol production

The molasses–CWP enzymatic hydrolysate (26.5% (w/v) initial total sugar) containing 0.8% w/v KH_2PO_4 and CWP solid residue at various concentration (0, 2.5, 3.0, 3.5 %, w/v) was fermented to ethanol for 48 hours. After centrifugation, supernatant was analysed for ethanol produced.

3.2.7 Ethanol production at optimized condition in 5L fermentor

Optimized molasses–CWP enzymatic hydrolysate was fermented to ethanol in 5L fermentor at 30°C, 100 rpm agitation without aeration for 48 hours. Culture obtained by centrifugation was analysed for ethanol produced.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Composition of cassava waste pulp

Major composition of cassava waste pulp (CWP) used in this study is shown in Table 2. Sriroth (Sriroth, Chollakup, Chotineeranat, Piyachomkwan, & Oates, 2000), Thongchul (Thongchul et al., 2010), and Soewarno (Soewarno, Primarini, & Sumaryono, 2012) reported that starch and protein content of CWP were 68.8, 50.0, 65.6% (w/w) and 1.6, 5.3, 3.1% (w/w), respectively. Since cassava waste pulp contained high carbohydrate and low protein contents. Therefore, it is suitable for ethanol production (Thongchul et al., 2010).

Table 2 *Composition of cassava waste pulp*

Components	% (w/w)*
Starch	67.8
Fat	1.5
Protein	2.1
Ash	3.7

*Food research and testing laboratory (FRTL), Faculty of Science, Chulalongkorn University

4.2 Cassava waste pulp hydrolysis

4.2.1 Acid hydrolysis

Cassava waste pulp hydrolyzed by HCl gave highest reducing sugar (0.284 g RS/g dry pulp) at 10% (w/v) substrate loading. Increasing of substrate loading to 20 and 30% (w/v) reduced reducing sugar yield to 0.269 and 0.209 (g RS/g dry pulp), respectively (Figure 18).

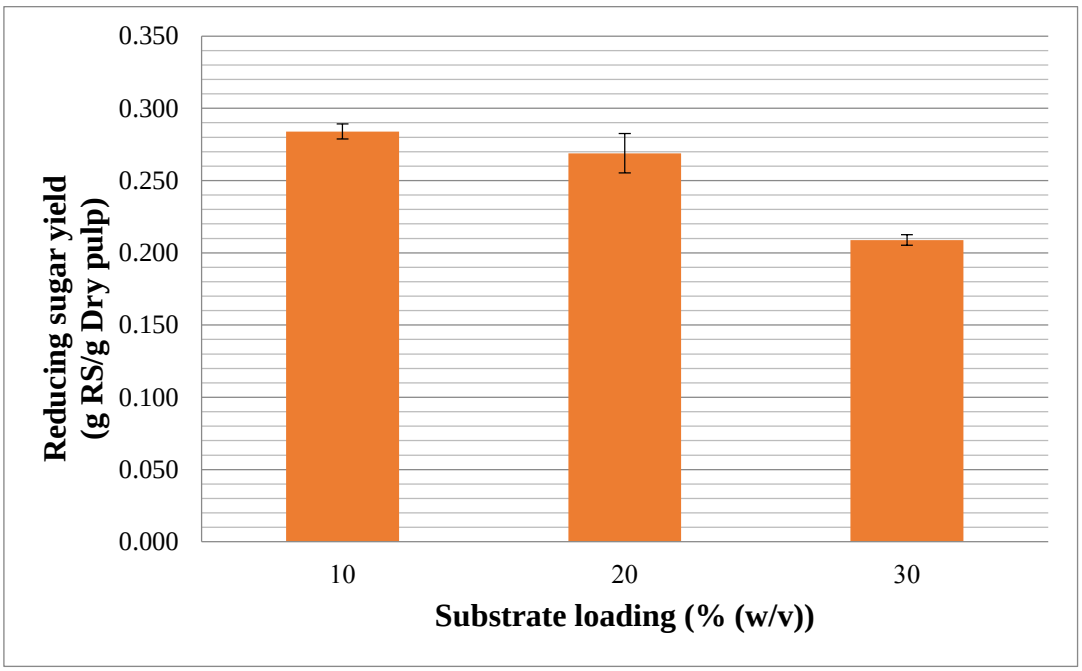


Figure 18 *Effect of substrate loading on reducing sugar yield from CWP- acid hydrolysis*

4.2.2 Enzymatic hydrolysis

Cassava waste pulp (25% and 50%, w/v substrate loading) sequentially hydrolysed by 3 enzymes; cellulase, α-amylase and glucoamylase; gave reducing sugar 0.140 and 0.096 (g RS/g dry pulp), respectively (Figure 19).

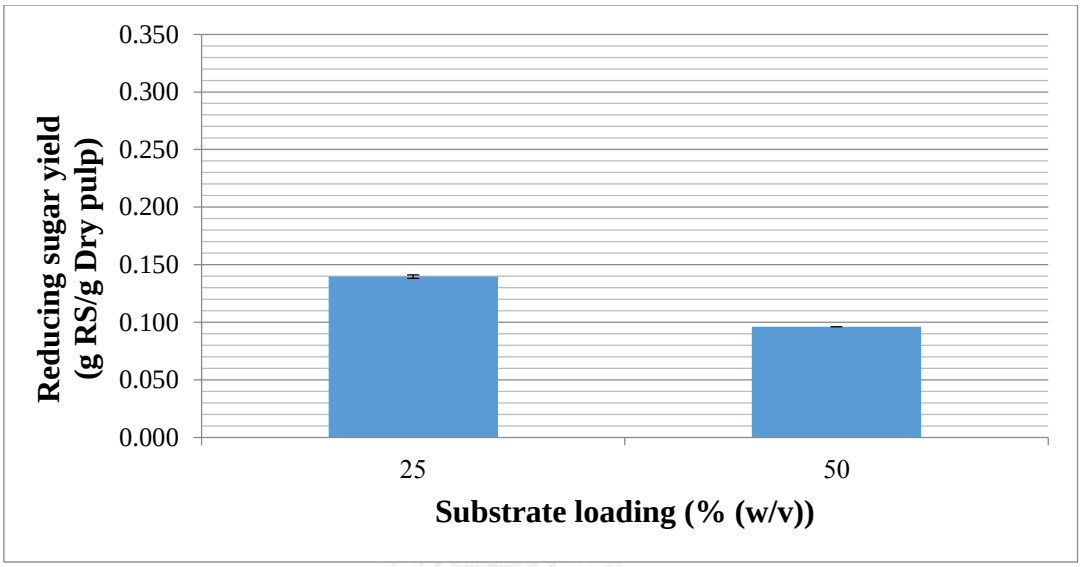


Figure 19 *Effect of substrate loading on reducing sugar yield from CWP-enzymatic hydrolysis*

Increasing of substrate loading reduced in liberation of reducing sugar from both acid and enzymatic hydrolysis of cassava waste pulp. Higher substrate loading reduced heat transfer during heat treatment (Fourier's law) and reduced surface area for substrate-enzyme/H^+ contact of both CWP- acid and enzymatic hydrolysis. Moreover, an enzyme activity might be inhibited by product in CWP- enzymatic hydrolysis.

4.3 Comparison of ethanol production from CWP-acid and CWP-enzymatic hydrolysate

CWP-acid and CWP-enzymatic hydrolysate obtained from the optimized condition in 4.2 supplemented with 0.2% (w/v) $(NH_4)_2SO_4$ were fermented to ethanol by *Saccharomyces cerevisiae* TISTR 5606 (SC 90). It was found that CWP-enzymatic hydrolysate gave 62.42% higher ethanol than CWP-acid hydrolysate (Figure 20). Acid hydrolysis of CWP might produced inhibitory compounds which inhibit enzyme activity important for growth and ethanol fermentation of yeast in subsequent step (Hatano et al., 2013; Modig et al., 2002).

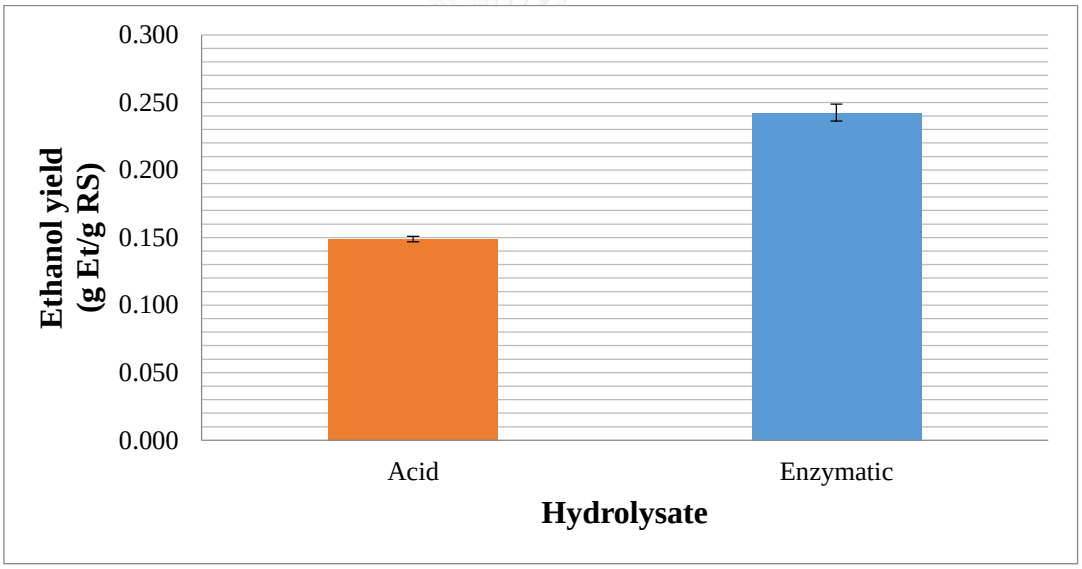


Figure 20 *Comparison of ethanol produced from CWP-acid and CWP-enzymatic* hydrolysate

4.4 Chemical composition of CWP–enzymatic hydrolysate

Chemical composition of CWP–enzymatic hydrolysate obtained from the optimized hydrolysis condition is shown in Table 3. It was found that the CWP-enzymatic hydrolysate contained many nutrients required for yeast growth and ethanol fermentation (Pradeep & Reddy, 2010; Takeshige & Ouchi, 1995). However, fermentable sugar content was too low to be used as substrate for ethanol production.

Table 3 *Chemical composition of CWP–enzymatic hydrolysate*

Components	**CWP–enzymatic hydrolysate*** **(g /100 g)**
Inorganic nutrients :	
Nitrogen(N)	Not detectable
Phosphorus(P)	6.82×10^{-3}
Potassium(K)	1.67×10^{-2}
Magnesium(Mg)	2.66×10^{-3}
Trace elements :	
Calcium(Ca)	1.79×10^{-3}
Copper(Cu)	$< 3.60 \times 10^{-4}$
Zinc(Zn)	1.10×10^{-4}
Manganese(Mn)	1.20×10^{-5}
Sugars :	
Sucrose	< 0.10
Glucose	2.84
Fructose	< 0.10

* Specific gravity of CWP–enzymatic hydrolysate was 1.01.

4.5 Chemical composition of molasses

Chemical composition of molasses is shown in Table 4.

Table 4 *Chemical composition of molasses*

Components	**Molasses** * **(g/100g)**
Inorganic nutrients :	
Nitrogen(N)	3.00×10^{-1}
Phosphorus(P)	1.20×10^{-1}
Potassium(K)	1.27
Magnesium(Mg)	2.30×10^{-1}
Trace elements :	
Calcium(Ca)	0.68
Copper (Cu)	$< 3.60 \times 10^{-4}$
Zinc (Zn)	1.70×10^{-4}
Manganese (Mn)	4.43×10^{-3}
Sugars :	
Sucrose	31.69
Glucose	8.73
Fructose	8.87
Volatile acid :	
Acetic acid	1.00
Non-volatile acid :	
Lactic acid	1.50

* Specific gravity of molasses was1.38**.**

The molasses was mixed with CWP-enzymatic hydrolysate to combine nutrients and fermentable sugar content. The molasses-CWP enzymatic hydrolysate mixture was fermented to ethanol by *S.cerevisiae* in further experiments.

4.6 Effect of nutrients supplementation in molasses–CWP enzymatic hydrolysate mixture on ethanol production

Molasses-CWP enzymatic hydrolysate supplemented with nutrients as shown in Table 3.1 were fermented to ethanol. Addition of 0.2% (w/v) KH_2PO_4 increased maximum ethanol production from 5.90% (w/v) to 6.27% (w/v) (Figure 21). This might due to molasses contained high concentration of Ca^{2+} (0.68% (w/v)) as shown in Table 4. In sugar production process, lime (CaO) is added to precipitate impurity of sugarcane juice. Ca^{2+} inhibited invertase enzyme (Chotineeranat et al., 2010). These residual Ca^{2+} form insoluble complex ($Ca_3(PO_4)_2$) with phosphate which is necessary for growth and metabolisms of yeast. (Chotineeranat et al., 2010; Takeshige & Ouchi, 1995). So, addition of KH_2PO_4 decreased inhibitory effect of Ca^{2+} and supplied nutrient (PO_4^{3-}) for yeast.

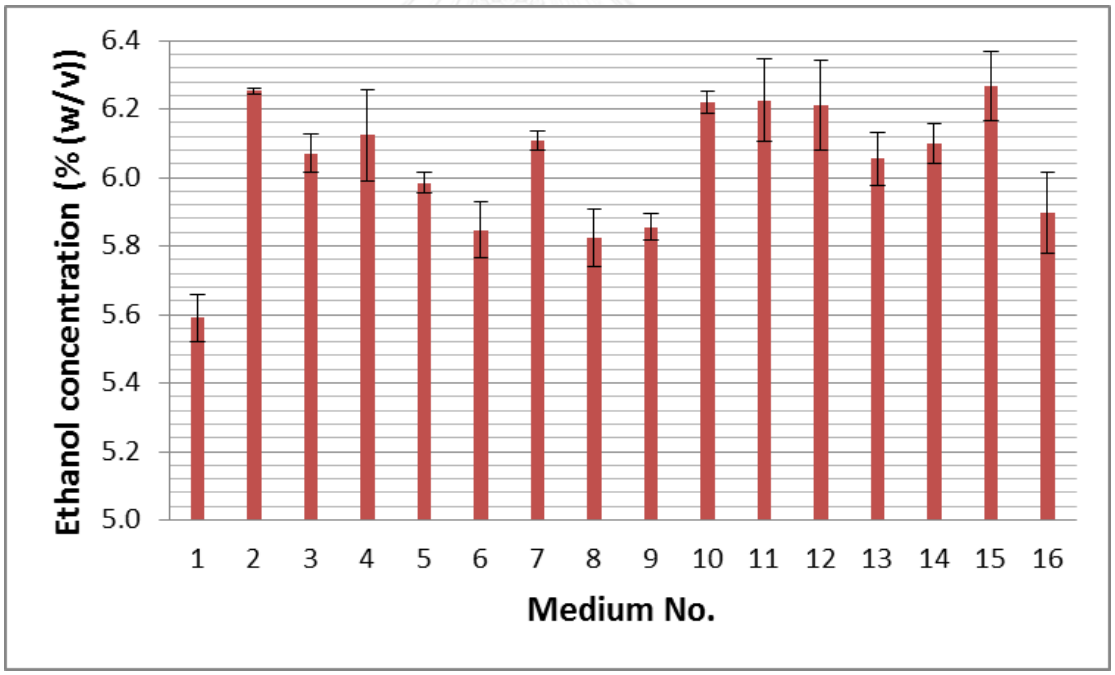


Figure 21 *Effect of nutrients supplementation on ethanol production from molasses–CWP enzymatic hydrolysate mixture*

4.7 Effect of KH_2PO_4 concentration in molasses–CWP enzymatic hydrolysate mixture on ethanol production

Fermentation of molasses–CWP enzymatic hydrolysate mixture contained various concentration of KH_2PO_4 showed that 0.8% (w/v) KH_2PO_4 gave highest ethanol (7.09% (w/v)) (Figure 22). Concentration of KH_2PO_4 after binding with Ca^{2+} to form $Ca_3(PO_4)_2$ complex was 0.6% (w/v).

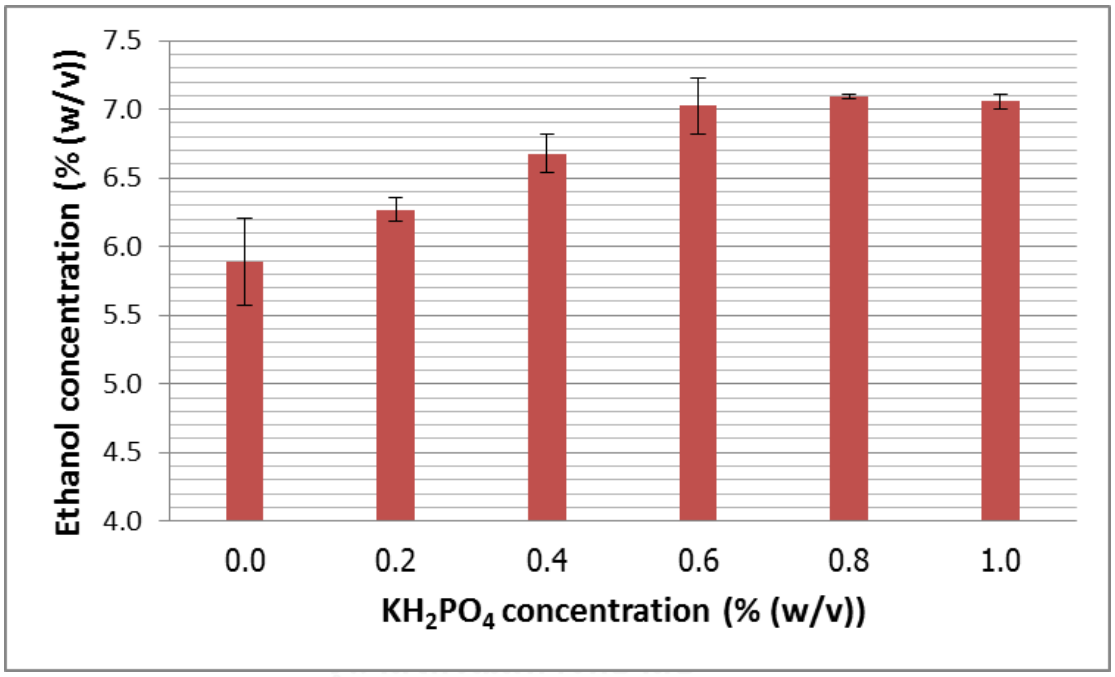


Figure 22 *Effect of KH_2PO_4 concentration on ethanol production from molasses–CWP enzymatic hydrolysate mixture*

4.8 Effect of initial total sugar concentration in molasses–CWP enzymatic hydrolysate mixture on ethanol production

Fermentation of molasses–CWP enzymatic hydrolysate mixture supplemented with 0.8% (w/v) KH_2PO_4 at various initial total sugar concentration revealed that 24.5 % (w/v) and 26.5 % (w/v) initial total sugar gave same maximal ethanol 7.06 and 7.09% (w/v) but ethanol yield were 0.31 and 0.29 g/g TS, respectively (Figure 23). *Saccharomyces cerevisiae* TISTR 5606 (SC 90) was under osmotic stress in 26.5% (w/v) initial total sugar (Limtong, 1997). Initial total sugar 24.5% (w/v) was used in next experiments.

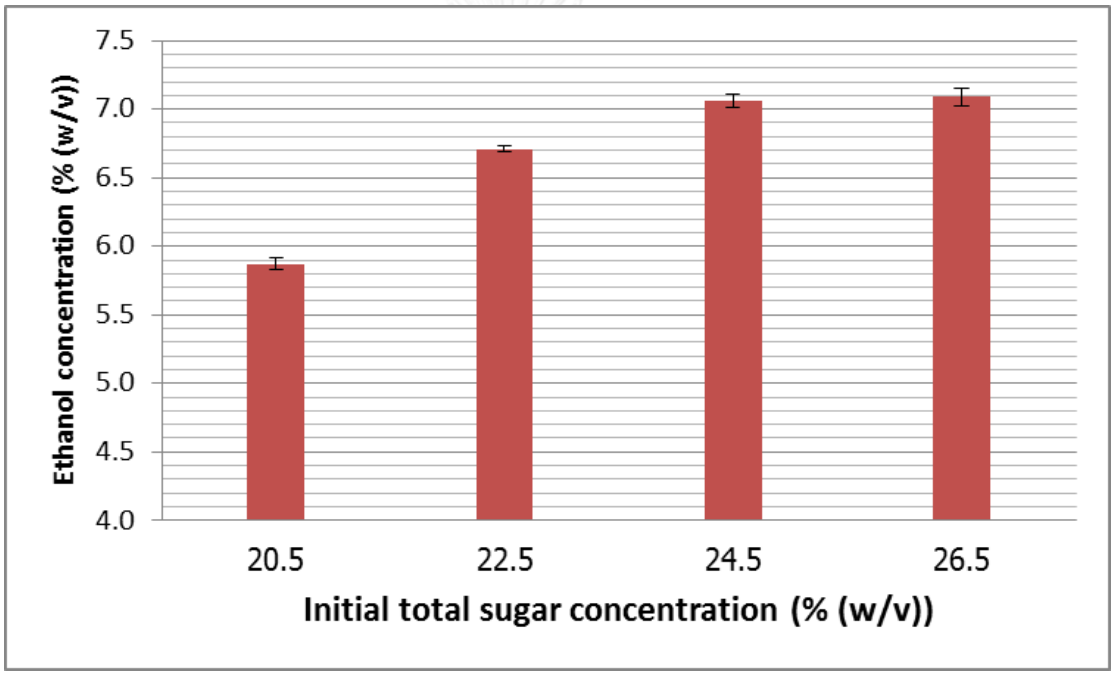


Figure 23 *Effect of initial total sugar concentration on ethanol production from molasses–CWP enzymatic hydrolysate mixture supplemented with 0.8% (w/v) KH_2PO_4*

4.9 Effect of KH_2PO_4& NaH_2PO_4 concentration in molasses–CWP enzymatic hydrolysate mixture on ethanol production

KH_2PO_4 is compose of K^+ and PO_4^{3-} which are both necessary for yeast in process of Na^+/K^+ ATP pump and synthesis of amino acid, nucleic acid, ATP and phospholipid (Ljungdahl & Daignan-Fornier, 2012; Pereira et al., 2010). So, ethanol production from molasses–CWP enzymatic hydrolysate mixture supplemented with various concentration of KH_2PO_4 or NaH_2PO_4 was investigated to determine for key supplement. It was found that maximum ethanol was obtained from molasses–CWP enzymatic hydrolysate mixture supplemented with both 0.8% (w/v) KH_2PO_4 and 0.8% (w/v) NaH_2PO_4. The above maximum ethanol produced were almost same level (Figure 24). So, PO_4^{3-} was concluded as a key supplement for ethanol production from molasses–CWP enzymatic hydrolysate mixture. Concentration for required PO_4^{3-} supplementation depend on concentration of Ca^{2+} in molasses because the Ca^{2+} forms insoluble complex with PO_4^{3-}.

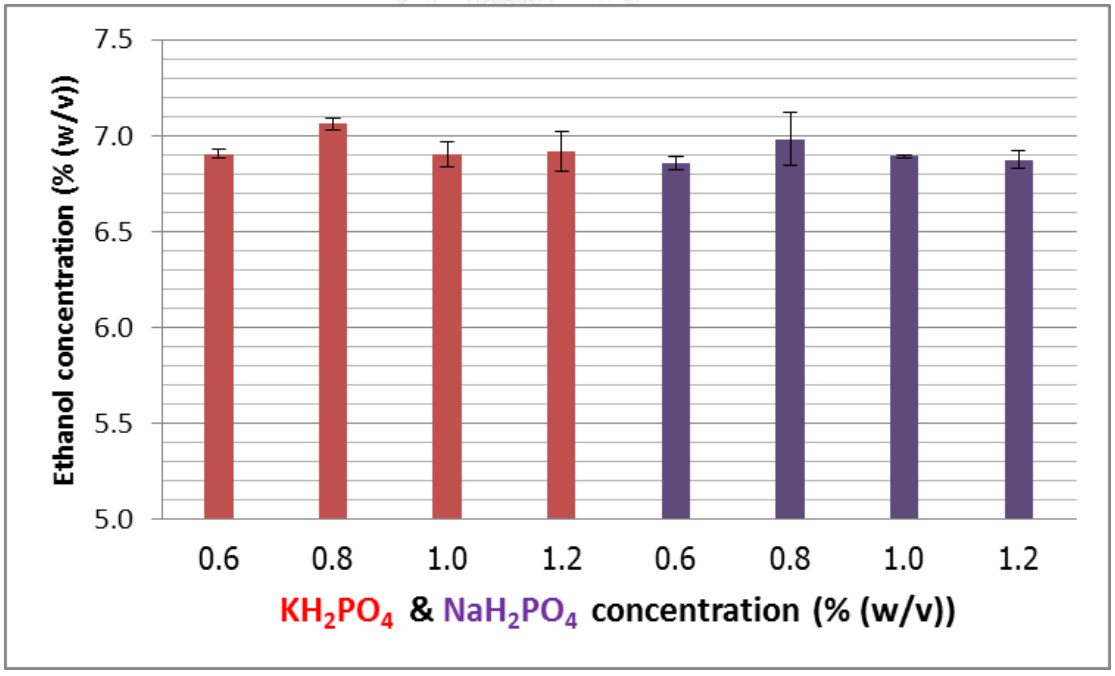


Figure 24 *Effect of KH_2PO_4& NaH_2PO_4concentration on ethanol production from molasses–CWP enzymatic hydrolysate mixture at 24.5% (w/v) initial total sugar*

4.10 Effect of CWP solid residue in molasses–CWP enzymatic hydrolysate mixture on ethanol production

CWP solid residue, 0, 2.5, 3.0 or 3.5% (w/v), was added into molasses–CWP enzymatic hydrolysate mixture supplemented with 0.8% (w/v) KH_2PO_4, then fermented to ethanol. It was found that in the presence of CWP solid residue, ethanol production increased. This might due to the CWP solid residue protected yeast cells from stresses such as ethanol toxicity, inhibitors and shearing force (Genisheva, Mussatto, Oliveira, & Teixeira, 2010; Holcberg & Margalith, 1981; Razmovski & Vučurović, 2011). Addition of 3% (w/v) of CWP solid residue gave the highest ethanol production at 7.44% (w/v) (Fig. 25). Addition of the CWP solid residue more than 3.5% (w/v) might obstruct nutrient transfer to yeast cell.

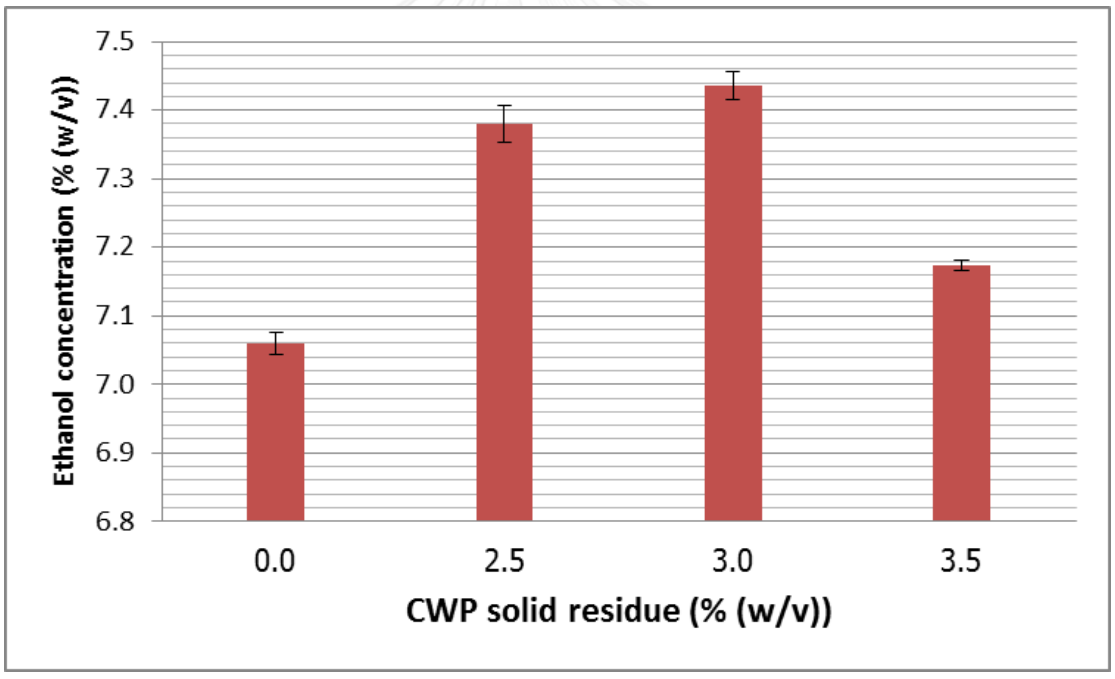


Figure 25 *Effect of CWP solid residue on ethanol production from molasses–CWP enzymatic hydrolysate mixture at 24.5% (w/v) initial total sugar supplemented with 0.8% (w/v) KH_2PO_4*

At optimal ethanol fermentation condition of molasses–CWP enzymatic hydrolysate mixture, highest ethanol production was 7.44% (w/v) and ethanol yield was 0.32 g/g TS. The low ethanol yield obtained might be a result of acetic acid contaminated in molasses. Acetic acid (>0.6% (w/v)) inhibited growth by increasing of cytoplasm pH and inhibiting of aldolase and triosephosphate isomerase activities (Narendranath, Thomas, & Ingledew, 2001). No fermentable sugars (glucose, fructose and sucrose) was detected in residual sugar (1.93%, w/v).

4.11 Ethanol production at the optimal condition in 5l fermentor

Fermentation of molasses–CWP enzymatic hydrolysate mixture at 24.5% (w/v) initial total sugar supplemented with 0.8% (w/v) KH_2PO_4 was upscaled from 50ml flask to 5L fermentor. The highest ethanol production in 5l fermentor was 7.58% (w/v) (0.31 g/g TS) at 48h (Figure 26). Ethanol production and ethanol productivity were 1.9% higher than in flask scale but ethanol yield was almost the same (0.32 and 0.31 g/g TS) in flask and in 5l fermentor, respectively.

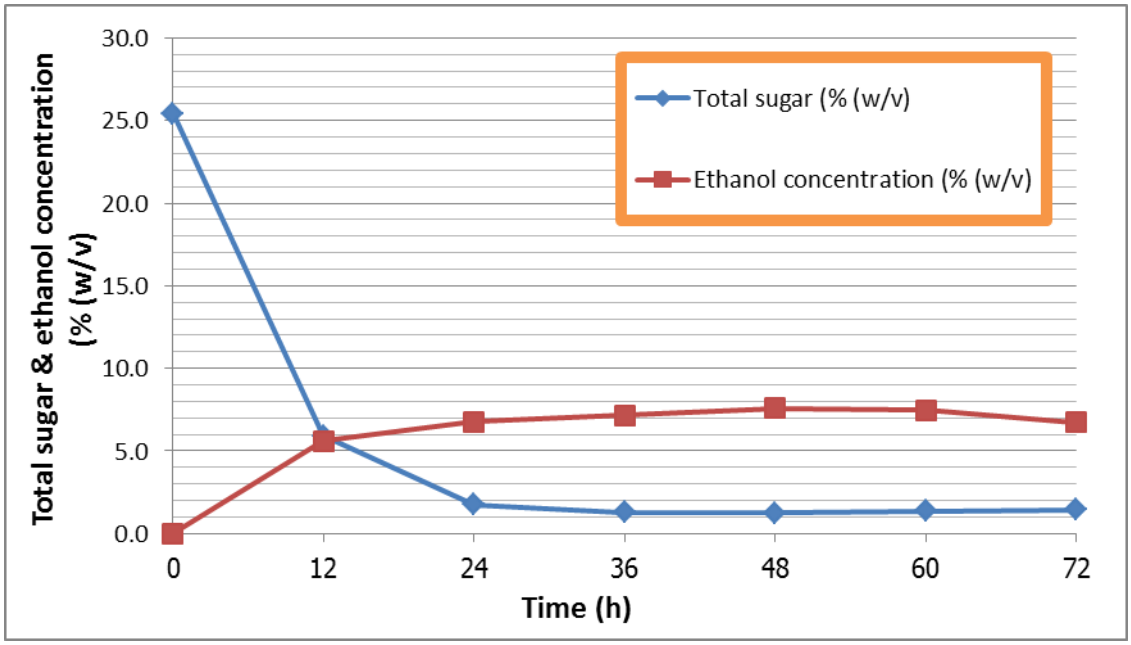


Figure 26 *Ethanol production from molasses–CWP enzymatic hydrolysate mixture at 24.5% (w/v) initial total sugar supplemented with 0.8% (w/v) KH_2PO_4 in 5l fermentor*

CHAPTER V

Conclusions

Value addition of cassava waste pulp (CWP) was investigated by co-fermentating with molasses. In this study, molasses (23.5%, w/v) was co-fermented with CWP enzymatic hydrolysate which contained 3% (w/v) CWP solid residue, 24.5% (w/v) initial total sugar using *Saccharomyces cerevisiae* TISTR 5606 (SC 90). Maximum ethanol produced at 48h was 7.44 and 7.58% (w/v) in flask and in 5l fermentor, respectively. When 0.8% (w/v) KH_2PO_4 was supplemented, ethanol productivity in 5l fermentor was 1.9% higher than in flask. Advantage of the co-fermentation was 1.) not necessary to add any nitrogen source, 2.) environmental problem caused by CWP was resolved and 3.) reduction in requirement of competitive substrate, molasses.

REFERENCES

Agriculture and Consumer Protection. Cassava processing. 2016, from http://www.fao.org/docrep/x5032e/x5032e04.htm

Agriculture and Consumer Protection. Renewable biological systems for alternative sustainable energy production
2016, from http://www.fao.org/ag/portal/index_en/en/

Ball, D. W., Hill, J. W., & Scott, R. J. Introduction to Chemistry: General, Organic, and Biological. 2016, from http://2012books.lardbucket.org/books/introduction-to-chemistry-general-organic-and-biological/index.html

Bhatia, L., Johri, S., & Ahmad, R. (2012). An economic and ecological perspective of ethanol production from renewable agro waste.

Brewer, M. Yeast Cell Counting and Viabilty: Hemocytometer. 2016, from http://microbebrewer.blogspot.com/2014/01/yeast-cell-counting-and-viabilty.html

Cao, T.-S., Chi, Z., Liu, G.-L., & Chi, Z.-M. (2014). Expression of TPS1 Gene from Saccharomycopsis fibuligera A11 in Saccharomyces sp. W0 Enhances Trehalose Accumulation, Ethanol Tolerance, and Ethanol Production. *Molecular biotechnology, 56*(1), 72-78.

Chotineeranat, S., Wansuksri, R., Piyachomkwan, K., Chatakanonda, P., Weerathaworn, P., & Sriroth, K. (2010). Effect of calcium ions on ethanol production from molasses by *Saccharomyces cerevisiae*. *Sugar Tech, 12*(2), 120-124.

Converti, A., Aliakbarian, B., Domínguez, J., Vázquez, G. B., & Perego, P. (2010). Microbial production of biovanillin. *Brazilian Journal of Microbiology, 41*(3), 519-530.

Cui, S. W. (2005). *Food carbohydrates: chemistry, physical properties, and applications*: CRC Press.

Danbamrongtrakool, N., Tungcharoen, T., & Parakulsuksatid, P. (2014). Effect of urea on ethanol production by *Saccharomyces cerevisiae* Sc90. *Proceedings of 52nd Kasetsart University Annual Conference: Agro-Industry*, 263-270.

Danon, B., Marcotullio, G., & de Jong, W. (2014). Mechanistic and kinetic aspects of pentose dehydration towards furfural in aqueous media employing homogeneous catalysis. *Green Chemistry, 16*(1), 39-54.

Deeprasert, S., Boonmunma, S., Boottongmol, W., Chinchoedwong, B., & Loha, V. (2011). Utilization of cassava soild waste for sugar production. *Thaksin University Journal, 15*(3), 39-46.

Fu, L., McCallum, S. A., Miao, J., Hart, C., Tudryn, G. J., Zhang, F., & Linhardt, R. J. (2015). Rapid and accurate determination of the lignin content of lignocellulosic biomass by solid-state NMR. *Fuel, 141*, 39-45.

Fujio, Y., Ogata, M., & Ueda, S. (1985). Ethanol fermentation of raw cassava starch with Rhizopus koji in a gas circulation type fermentor. *Biotechnology and bioengineering, 27*(8), 1270-1273.

Genisheva, Z., Mussatto, S. I., Oliveira, J., & Teixeira, J. (2010). Selection of natural material for Saccharomyces cerevisiae immobilization. *Journal of Biotechnology, 150*, 399-400.

Gnansounou, E., & Dauriat, A. (2005). Ethanol fuel from biomass: A review. *Journal of Scientific and Industrial Research, 64*(11), 809.

Goyal, A., Ghosh, B., & Eveleigh, D. (1991). Characteristics of fungal cellulases. *Bioresource technology, 36*(1), 37-50.

Hatano, K.-i., Aoyagi, N., Miyakawa, T., Tanokura, M., & Kubota, K. (2013). Evaluation of nonionic adsorbent resins for removal of inhibitory compounds from corncob hydrolysate for ethanol fermentation. *Bioresource technology, 149*, 541-545.

Hatano, K.-i., Kikuchi, S., Nakamura, Y., Sakamoto, H., Takigami, M., & Kojima, Y. (2009). Novel strategy using an adsorbent-column chromatography for effective ethanol production from sugarcane or sugar beet molasses. *Bioresource technology, 100*(20), 4697-4703.

Holcberg, I., & Margalith, P. (1981). Alcoholic fermentation by immobilized yeast at high sugar concentrations. *European journal of applied microbiology and biotechnology, 13*(3), 133-140.

Limtong, S. (1997). *Yeast and yeast technology microbiology*: KU Press.

Ljungdahl, P. O., & Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in Saccharomyces cerevisiae. *Genetics, 190*(3), 885-929.

Modig, T., Lidén, G., & Taherzadeh, M. J. (2002). Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal, 363*(Pt 3), 769-776.

Naik, S., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: a comprehensive review. *Renewable and Sustainable Energy Reviews, 14*(2), 578-597.

Najafpour, G., Younesi, H., & Ismail, K. S. K. (2004). Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. *Bioresource technology, 92*(3), 251-260.

Narendranath, N., Thomas, K., & Ingledew, W. (2001). Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. *Journal of Industrial Microbiology and Biotechnology, 26*(3), 171-177.

Ng, T. K., Ben-Bassat, A., & Zeikus, J. (1981). Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of Clostridium thermocellum and Clostridium thermohydrosulfuricum. *Applied and Environmental Microbiology, 41*(6), 1337-1343.

NSW Sugar Milling Co-operative. Processing Sugar Cane into Raw Sugar. 2016, from http://people.uwec.edu/ivogeler/travel/cuba/sugar-cane.htm

Office of the national economic and social development board. (2006). *Final report : Zero Waste Industry project*.

Patil, S., Premalatha, B., Rao, R. S., & Ganavi, B. (2013). Revelation in the field of tissue preservation-A preliminary study on natural formalin substitutes. *Journal of International Oral Health, 5*(1), 31.

Pereira, F. B., Guimarães, P. M., Teixeira, J. A., & Domingues, L. (2010). Optimization of low-cost medium for very high gravity ethanol fermentations by Saccharomyces cerevisiae using statistical experimental designs. *Bioresource technology, 101*(20), 7856-7863.

Pradeep, P., & Reddy, O. (2010). High gravity fermentation of sugarcane molasses to produce ethanol: Effect of nutrients. *Indian journal of microbiology, 50*(1), 82-87.

Punnapayak, H., & Prasongsuk, S. (2015). *Industrial botany*: CU Press.

Razmovski, R., & Vučurović, V. (2011). Ethanol production from sugar beet molasses by S. cerevisiae entrapped in an alginate–maize stem ground tissue matrix. *Enzyme and microbial technology, 48*(4), 378-385.

Riansa-ngawong, W., Suwansaard, M., & Prasertsan, P. (2012). Application of palm pressed fiber as a carrier for ethanol production by Candida shehatae TISTR5843. *Electronic Journal of Biotechnology, 15*(6), 1-1.

Ruangviriyachai, C., & Niwaswong, C. (2012). Production of cellulosic ethanol in Thailand. *KKU Science Journal, 40*(4), 1073-1088.

Saey, T. H. First chromosome made synthetically from yeast. 2016, from https://www.sciencenews.org/article/first-chromosome-made-synthetically-yeast

Soewarno, N., Primarini, D., & Sumaryono, W. (2012). CASSAVA PULP AS A BIOFUEL FEEDSTOCK OF AN ENZYMATIC HYDROLYSIS PROCESS. *MAKARA of Technology Series, 15*(2).

Somogyi, M. (1952). Determination of reducing sugars by Nelson–Somogyi method. *J. Biol. Chem, 200*, 245.

Sriroth, K., Chollakup, R., Chotineeranat, S., Piyachomkwan, K., & Oates, C. G. (2000). Processing of cassava waste for improved biomass utilization. *Bioresource technology, 71*(1), 63-69.

Sriroth, K., & Piyachomkwan, K. (2007). *Starch technology*: KU Press.

Subramaniam, B., Zuo, X., Busch, D. H., & Venkitasubramanian, P. (2012). Spray oxidation process for producing 2, 5-furandicarboxylic acid from hydroxymethylfurfural: Google Patents.

Takeshige, K., & Ouchi, K. (1995). Factors affecting the ethanol productivity of yeast in molasses. *Journal of fermentation and Bioengineering, 79*(5), 449-452.

Tesfaw, A., & Assefa, F. (2014). Current trends in bioethanol production by Saccharomyces cerevisiae: substrate, inhibitor reduction, growth variables, coculture, and immobilization. *International Scholarly Research Notices, 2014*.

Thongchul, N., Navankasattusas, S., & Yang, S.-T. (2010). Production of lactic acid and ethanol by Rhizopus oryzae integrated with cassava pulp hydrolysis. *Bioprocess and biosystems engineering, 33*(3), 407-416.

Watanabe, I., Miyata, N., Ando, A., Shiroma, R., Tokuyasu, K., & Nakamura, T. (2012). Ethanol production by repeated-batch simultaneous saccharification and fermentation (SSF) of alkali-treated rice straw using immobilized Saccharomyces cerevisiae cells. *Bioresource technology, 123*, 695-698.

Wilaithup, A., Laemsak, N., Sirisansaneeyakul, S., Vanichsriratana, W., & Parakulsuksatid, P. (2013). Effect of glucose concentrations on ethanol production by Saccharomyces cerevisiae Sc90. *Proceedings of 51st Kasetsart University Annual Conference: Agricultural Extension and Home Economics, Agro-Industry*, 123-129.

APPENDIX

APPENDIX A

Culture medium

1. Yeast extract peptone dextrose agar (YPD agar)

Glucose	20	g
Peptone	20	g
Yeast extract	10	g
Agar	20	g
Distilled water	1000	ml

pH adjusted to 5.5 and steriled by autoclaving at 121°C for 15 min.

2. Yeast extract peptone dextrose broth (YPD broth)

Glucose	20	g
Peptone	20	g
Yeast extract	10	g
Distilled water	1000	ml

pH adjusted to 5.5 and steriled by autoclaving at 121°C for 15 min.

3. Fermentation medium

$(NH_4)_2SO_4$	2	g	
KH_2PO_4	2	g	
$MgSO_4.7H_2O$	0.75	g	
Yeast extract	10	g	
Molasses	x	g	(up to initial sugar concentration)
CWP hydrolysate	1000	ml	

pH adjusted to 5.5 and steriled by autoclaving at 110°C for 10 min.

APENDIX B

Reagents and analysis methods

1. Somogyi-Nelson method (Somogyi, 1952)

1.1 Somogyi-Nelson reagents

1.1.1 Alkaline copper reagent

$Na_2HPO_4 \cdot 12H_2O$	71	g
$KNaC_4H_4O_6 \cdot 5H_2O$	40	g
1N NaOH	100	ml
10% (w/v) $CuSO_4 \cdot 5H_2O$	80	ml

(Dissolve 8 g $CuSO_4 \cdot 5H_2O$ in 80 ml distilled water)

Na_2SO_4	180	g

*Adjust volume to 1000 ml by distilled water *

1.1.2 Nelson's reagent

$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	53.2	g
Conc. H_2SO_4	21	ml
12% (w/v) $Na_2HAsO_4 \cdot 7H_2O$	50	ml

(Dissolve 6 g $Na_2HAsO_4 \cdot 7H_2O$ in 50 ml distilled water)

*Adjust volume to 1000 ml by distilled water *

1.2 Analysis method

1 ml sample (Blank : distilled water)

↓

Add 1 ml Alkaline copper reagent

↓ Vortex

↓ Boil for 15 min.

↓ Cool immediately

Add 1 ml Nelson reagent

↓ Vortex

↓ Incubate at RT for 30 min.

Add 5 ml distilled water

↓ Vortex

Measure the absorbance at 520 nm.

Standard curve of Somogyi-Nelson method was prepared from absorbance of 0, 20, 40, 60, 80, 100, 120, 150, 180 and 200µl/ml glucose solution (glucose stock solution 1 mg/ml) plot against concentration of glucose solution

1.3 Standard curve of Somogyi-Nelson method

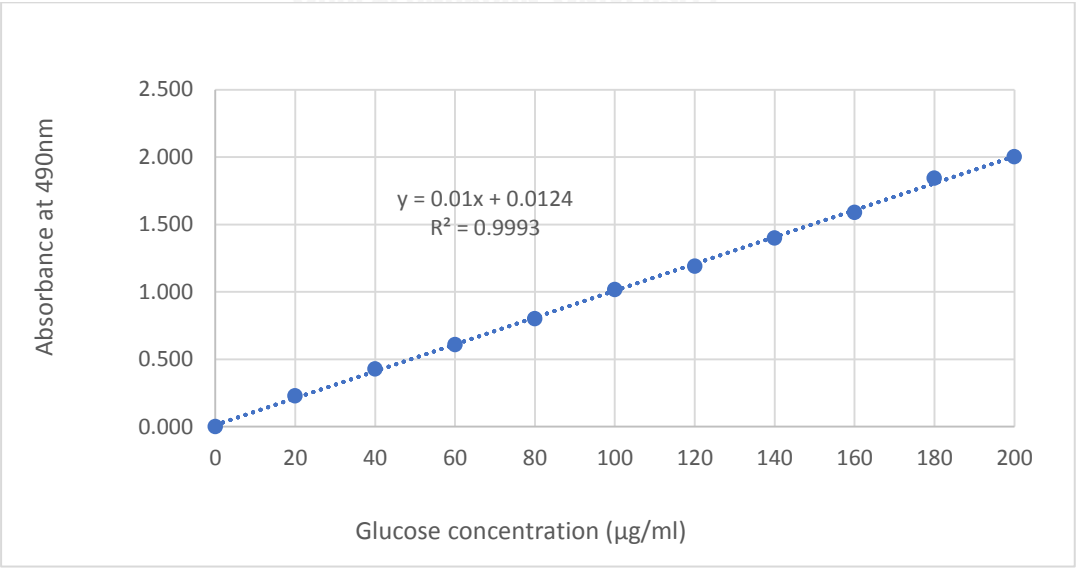


Figure 27 *Standard curve of Somogyi-Nelson method*

2. Phenol-sulfuric method (Dubois, 1958)

2.1 Phenol-sulfuric reagents

2.1.1 5% (w/v) Phenol

Phenol	5	g
Distilled water	100	ml

2.1.2 Conc. H_2SO_4

2.2 Analysis method

1 ml sample (Blank : distilled water)

↓

Add 1ml 5% (w/v) Phenol

↓ Vortex

Add 5 ml Conc. H_2SO_4

↓ Incubate at RT for 30 min.

↓ Vortex

Measure the absorbance at 490 nm.

Standard curve of Phenol-sulfuric method was prepared from absorbance of 0, 20, 40, 60, 80, 100, 120, 150, 180 and 200µl/ml sucrose solution (sucrose stock solution 1mg/ml) plot against concentration of sucrose solution

2.3 Standard curve of Phenol-sulfuric method

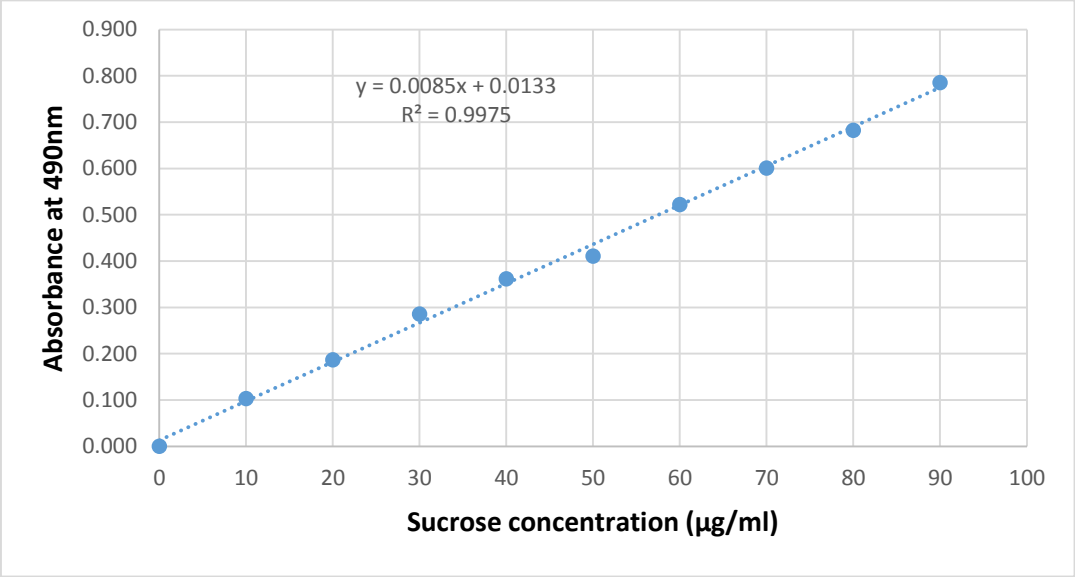


Figure 28 *Standard curve of Phenol-sulfuric method*

APPENDIX C

Cell counting

1. Cell counting with a hemocytometer

Clean both the hemocytometer and cover slip

↓

Place the cover slip over counting surface

↓

Load sample

↓

Place on microscope stage

↓

Adjust focus of counting grid

↓

Count cell in each highlighted boxes

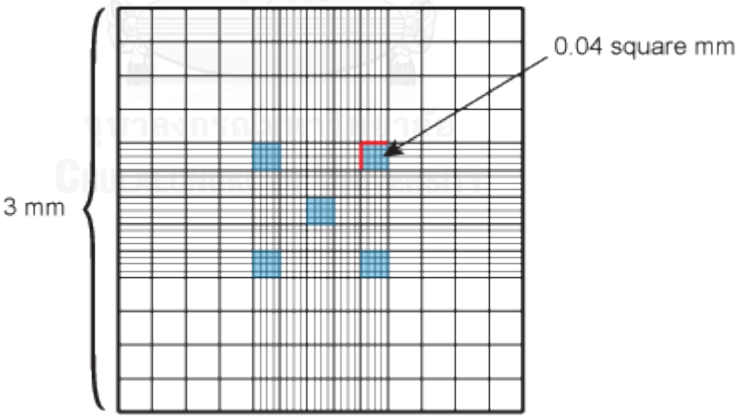


Figure 29 *Counting grid of hemocytometer*
(Brewer, 2016 : online)

2. Calculation

Volumn of a highlighted box = Width x Length x Depth

= 0.2 mm x 0.2 mm x 0.1 mm

= $4.0 \times 10^{-3}(mm)^3$

= 4.0×10^{-6}ml

The average cell number = $\frac{\text{Cell number of five highlighted boxes}}{\text{Number of highlighted boxes}}$

= A cells

Cell concentration = $\frac{\text{A cells}}{4.0 \times 10^{-6}\ \text{ml}}$

VITA

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