CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Biobutanol as a Biofuel

2.1.1 Properties of Biobutanol

Butanol is an alcohol, which consists of four carbon atoms and ten hydrogen atoms with a molecular weight of 74.12. Because of the properties of butanol, it has been widely used as a biofuel (liquid transportation fuel). The relevant properties of n-butanol compared with other liquid fuels are presented in Table 2.1.

Table 2.1 Relevant properties of n-butanol compared with other liquid fuels (http://www.eolss.net/Sample-Chapters/C17/E6-58-05-11.pdf)

Property	Gasoline	Ethanol	Butanol	Biodiesel	Diesel
Reid Vapour Pressure (Psi)	62	15	2.3	-	<3
Lower Flamibility limit					
Concentration (Vol%)	1.4	3.3	1.4	-	0.6
Temperature (°C)	-45	13	36	-	64
Upper Flamibility limit					
Concentration (Vol%)	7.6	19	11.2	-	5.6
Temperature (°C)	-20	42	-	-	150
Flash Point (°C)	-43	13	36	>120	64
Autoignition Temperature (°C)	300	366	343	-	230
Cloud Point (°C)	NA	NA	-89	0	-26
Density (kg/l)	0.791	0.785	0.81	0.86	0.863
Vapour Specific Gravity	3.5	1.6	2.6	-	5.5
Kinematic Viscosity (mm^2/sec)	NA	NA	3.7	3.5-5	2-8
Lower Heating Value					
Mass (MJ/kg)	43.9	27.0	33.22	37.8	42.6
Volume (MJ/L)	32.7	21.2	26.9	32.5	36.7
BTU per Gallon	115,000	110,000	84,000	120,000	13,000

Property	Gasoline	Ethanol	Butanol	Biodiesel	Diesel
Research Octane Number	90–100	108	96	NA	NA
Cetane Number	NA	2–12	17	>51	40–47.5

There are many interesting comparisons between butanol and ethanol. Butanol has more energy density per litre than ethanol for 25 % and energy contents of butanol and ethanol are composed of 90 % and 60 % BTU per gallon, respectively. Higher energy content gives possible premium biofuel applications by charging more at the pump for a superior fuel. Although butanol production requires higher cost than ethanol, the higher performance in engines from butanol causes better mileage. A further energy advantage in the production of biobutanol provides 18 % more energy produced from the same amount of fermentable substrate as ethanol.

Furthermore, butanol has a similar octane rating to gasoline but not as high as that of ethanol and it doesn't cause the toxicity problems of Methyl Tertiary Butyl Ether (MTBE). The vapour pressure of butanol is 0.33, while others have 2.00 for ethanol and 4.50 for gasoline. The low vapour pressure point coupled with a high flash point makes butanol safer to use at high temperatures. Next, butanol has a great tolerance to moisture and water contamination because of very low affinity for water (7.8 %) in comparison with ethanol (100 %), thus butanol grants low corrosive and is easier to handle than ethanol or gasoline.

The performance of butanol as transportation fuel has been widely recognized for a long time. Butanol is well suited with current vehicle and engine technologies and also supports environmental regulation. In addition, butanol has the usage ratio of fuel to air which are closer to that of gasoline than ethanol. It can be blended directly with gasoline and transported via existing gasoline pipelines, in the meantime currently ethanol has to be transported separately and mixed at the fuel outlet. Ethanol has a limitation for mixing before internal combustion for around 10 %; hence engine modifications are required. On the other hand, butanol can be blended into gasoline at higher concentrations without modification the vehicles due to low vapor pressure (Amrita and Moholkar, 2009).

The attractive features of biobutanol are using as a co-blending agent with ethanol and gasoline and can be total replacements for gasoline, diesel, and possibly aviation and jet fuels. Currently, biobutanol can be mixed up to 10 %v/v in European gasoline as well as 11.5 %v/v in US gasoline. Enhancing the performance of the fuel blends in this way could rapidly growth of the overall biofuels market along with the development of agricultural markets.

2.2 Lignocellulosic-biomass Materials

Lignocellulosic biomass from forestry, agricultural, and agro-industrial wastes have been recognized as abundant, renewable, and inexpensive energy sources. Wastes are composed of a various materials as the following; switchgrass, straws, sugarcane bagasse, leaves, husks, wheat, corn, sorghum, barley, sawdust, and etc. With a number of accumulations of lignocellulose wastes every year, they caused the environmental problems. Nevertheless, a number of valued products from wastes such as ethanol, food additives, organic acids, enzymes, and others could be utilized for the production because their chemical compositions based on sugars and other compounds of interest.

Figure 2.1 shows the major components of lignocellulose, which are closely connected with each other constituting the cellular complex of the vegetal biomass, are cellulose, hemicellulose, and lignin. Besides, the structure of lignocellulose is presented as surrounded cellulose forms by hemicellulose and lignin.

The content of cellulose, hemicelluloses, and lignin in common agricultural residues are listed in Table 2.2.

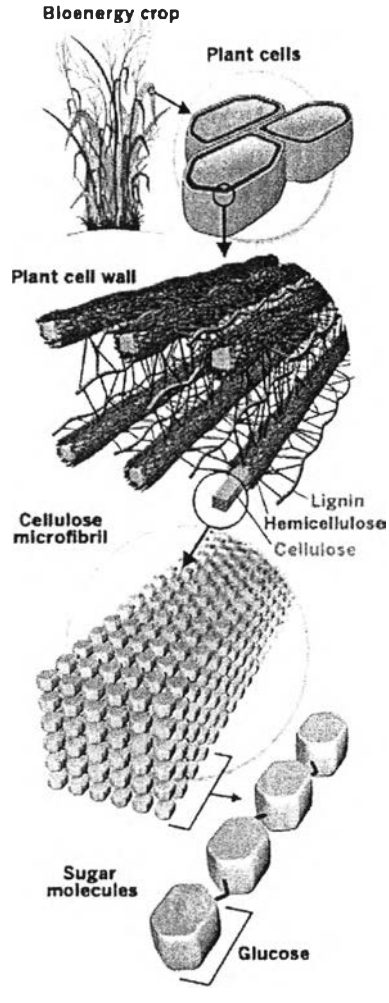

Figure 2.1 Major components of lignocelluloses.
(http://www.secondgenome.com/2010/08/lignocellulose-decomposition)

Table 2.2 The content of cellulose, hemicelluloses, and lignin in common agricultural residues and wastes (Sun and Cheng, 2002)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corncobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15

Table 2.2 The content of cellulose, hemicelluloses, and lignin in common agricultural residues and wastes (Sun and Cheng, 2002) (cont.)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA	24–29
Swine waste	6.0	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

2.2.1 Cellulose

Cellulose is a linear homopolymer with repeated units of cellobiose (two anhydrous glucose rings joined via a β-1,4 glycosidic linkage), which has high molecular weight. Cellulose was packed into microfibrils because of linked long-chain cellulose polymers with hydrogen and van der Walls bonds. Due to forming of hydrogen bounds, this chain was arranged in parallel and form crystalline structure as highly crystalline regions (around 2/3 of the total cellulose) and less-ordered amorphous regions. In addition, solubility and degradation are decreased with more ordered or crystalline cellulose. Structure of celluose chain is shown in Figure 2.2.

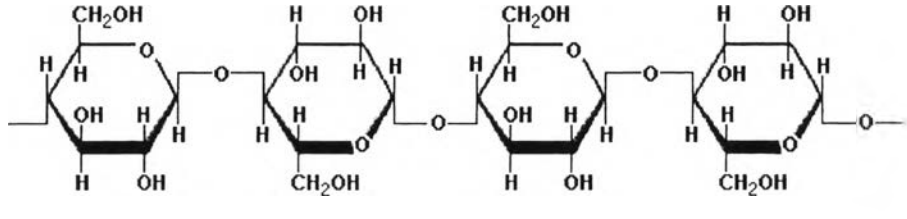

Figure 2.2 Structure of cellulose chain.
(http://www.scientificpsychic.com/fitness/carbohydrates2.html)

2.2.2 Hemicellulose

Hemicellulose is a linear and branched heterogeneous polymer including five different sugars: D-Larabinos, D-galactose, D-glucose, D-mannose, and D-xylose regularly with other components such as acetic, ferulic, and glucuronic acids. The backbone of the chains of hemicelluloses can be either homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars). Refer to the main sugar residue in the backbone, hemicellulose can be classified such as xylans (Figure 2.3), mannans, glucans, glucuronoxylans, arabinoxylans (Figure 2.4), glucomannans, galactomannans, galactoglucomannans, β-glucans, and xyloglucans. Hemicelluloses structure is easier to hydrolyze than cellulose because of composition of sugar units, presence of shorter chains a branching of main chain molecules, and to be amorphous.

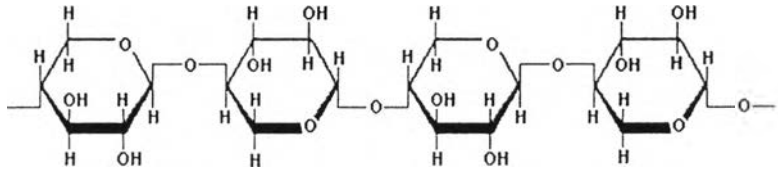

Figure 2.3 Structure of xylan.
(http://www.scientificpsychic.com/fitness/carbohydrates2.html)

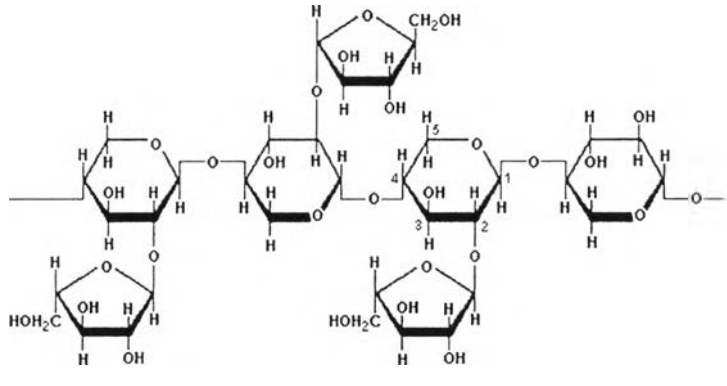

Figure 2.4 Structure of arabinoxylan.
(http://www.scientificpsychic.com/fitness/carbohydrates2.html)

2.2.3 Lignin

Lignin has been commonly realized as a very complex molecule, which constructed of phenylpropane units linked in a large three-dimensional structure. Three phenyl propionic alcohols, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, are known as monomers of lignin (Figure 2.5). Lignin function with bounding to cellulose and hemicelluloses is to equip rigidity and cohesion to the material cell wall, to confer water impermeability to xylem vessels, and to form a physic–chemical barrier against microbial attack. Resistant to chemical and enzymatic degradation of lignin's molecular configuration are clearly excellent.

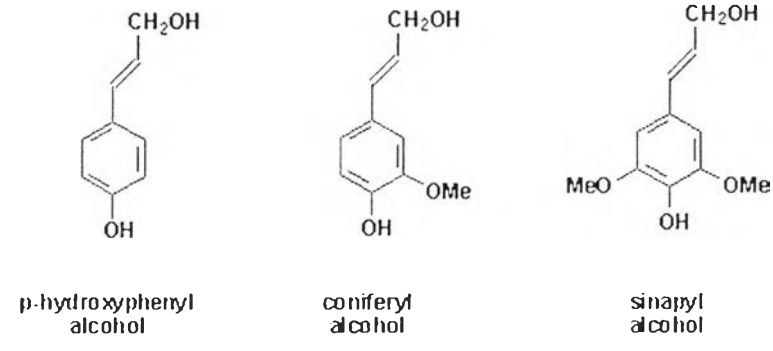

Figure 2.5 Structure of monolignols.
(http://www.helsinki.fi/agriculturalsciences/research/Gerberalab/lignin_monolignols.html)

2.3 Pretreatment of Lignocellulosic Biomass

There are many purposes of the pretreatment such as removing hemicellulose and lignin, reducing cellulose crystallinity, and increasing the porosity of the materials. Figure 2.6 presents characteristics of lignocellulosic material after pretreatment process meets the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective (Sun and Cheng, 2002). Several pretreatment processes used pretreatment additives, biomass and energy mechanical heat as raw materials, while solid residues

consisting of cellulose hemicellulose and lignin are main product, as well as other products are form of vapour and liquid phase. There are many types of pretreatment process such as physical, physico–chemical, chemical, and biological processes; however, this investigation concentrates on using dilute acid and enzyme hydrolysis as chemical pretreatment.

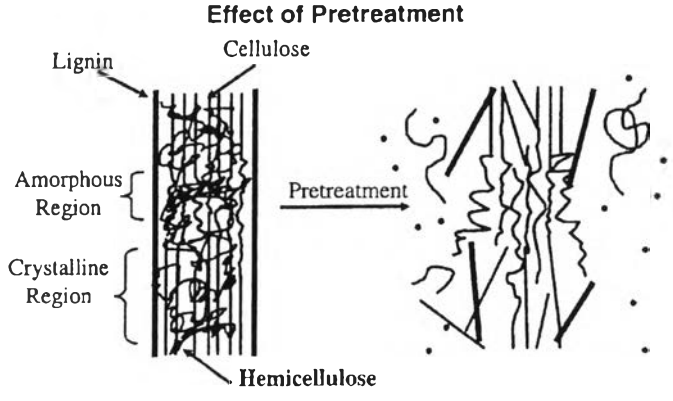

Figure 2.6 Schematic of goals of pretreatment on lignocellulosic material (Balat, 2011).

2.3.1 Dilute Acid Pretreatment

The cellulose and hemicelluloses polymers in lignocellulosic biomass can be broken down by dilute or concentrated acids into individual sugar molecules form, which will be biofuel after fermentation. It was found that hemicellulose is more easily hydrolysed than cellulose.

The major objective of the acid pretreatment is to make the cellulose more accessible to enzymes and to solubilize the hemicellulosic fraction of the bio-mass. This pretreatment can be achieved by concentrated or dilute acid, but owing to the formation of inhibiting compounds, the application of concentrated acid is less attractive for ethanol production.

Besides, using concentrated acid pretreatments was realized some disadvantages that caused equipment corrosion problems and acid recovery. The applying the concentrated acid pretreatment at commercial scale reduces the interest with high operational and maintenance costs.

Dilute acid pretreatment has been successful development for pretreatment of lignocellulosic materials. With dilute sulfuric acid pretreatment, it significantly improved cellulose hydrolysis and achieved high reaction rates (Esteghlalian *et al.*, 1997). At moderate temperature, direct saccharifacation suffered from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis (MacMillan, 1994). According to xylan is accounted for up to a third of the total carbohydated in many lignocellulosic material, achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics (Sun and Cheng, 2002). Moreover, dilute sulfuric acid pretreatment has been studied and widely used because it is inexpensive and effective process (Zheng *et al.*, 2009). The amount of xylan oligomers as a fraction of the total solubilized xylose was greatest at short pretreatment times, coinciding with the breakdown of the fast xylan fraction (Esteghlalian *et al.*, 1997). With too long residence time and so high reaction temperature, carbohydrates are degraded into furfural and HMF which in turn are degraded into levulinic acid and formic acid, respectively (Redding *et al.*, 2011). Glucose in the hydrolyzate step would be influenced most substantially by temperature and acid concentration. For using corncobs as a substrate, in our group we found that the optimal condition is at 120 °C with a sulfuric acid concentration of 2 % and 5 minute reaction time. This condition was released the total sugar around 47.11 g/l (Tangmanasakul, 2011). Some research worked on rice straw (Kim *et al.*, 2012) or wheat straw (Baboukani *et al.*, 2012). It was found that maximum solubilized xylose are 73.14 % and 69.0452 % which can obtain at 1.21% acid, 142 °C for 11.6 min and 1.53 % acid, 147 °C for 30 min, respectively.

One of the most widely used acids is phosphoric acid because phosphoric acid is less aggressive than other acids which give solution higher concentration of growth inhibitors of microorganism such as furfural or acetic acid (Romero *et al.*, 2007) and after neutralization of hydrolysates with NaOH, the salt formed is sodium phosphate. This salt can remain in the hydrolysates because it is used as nutrient by microorganisms. Therefore, an operation of filtration is not needed with the consequent advantage: improve the economics of the process (avoid the filtration to remove the salts and decrease the amount of nutrient needed for fermentation) and

is friendly with the environment (the salt formed is not waste) (Gámez *et al.*, 2006). Dilute phosphoric acid was used to remove hemicellulose and lignin in order to increase reducing sugar in our previous work (Satimanont, 2012). In pretreatment step, while harsher pretreatment conditions (> 120 °C), the xylose yield decreased owing to xylose degradation into furfural. Pretreatment temperature and time can drive xylose degradation into furfural and furfural formation is faster at higher temperatures and times. For hydrolysis step, the glucose yield increased with an increase of pretreatment temperature and time. Furfural formation is influenced by temperature and acid concentration so it can imply that the glucose in hydrolysate would be influenced by temperature and acid concentration as well. In this research, the overall highest total sugar yield in both pretreatment and enzymatic hydrolysis was 46.14 g/L under an condition at 140 °C for 10 min of pretreatment time using 2% (w/w) H_3PO_4 and a 10:1 liquid to solid ratio. Gómez *et al.* has found that sulfuric acid pretreatment at best compared with phosphoric acid, as it allowed higher yields in lower reaction times and concentrations by using Response Surface Methodology when sugarcane is a feedstock. Other acid have also been studied, such as hydrochloric acid and nitric acid.

Dilute acid pretreatment was selected as the preferable method for industrial applications, and also has been widely learned about lignocellulosic biomass. This pretreatment of lignocellulosic biomass has been applied by different types of reactors such as percolation, plug flow, shrinking-bed, batch, and countercurrent reactors, which can be performed both at high temperature during a short period of time (e.g. 180 °C), or at lower temperature for longer retention time (e.g. 120 °C for 30–90 min).

Even if the advantage of solubilizing hemicellulose, mainly xylan, was presented, it was also converted to fermentable sugars. However, it was found that some sugar degradation compounds such as furfural and HMF and aromatic lignin degradation compounds affected the microorganism metabolism in the fermentation step. Furthermore, degradation product was originated lower than concentrated acid pretreatments.

2.4 Lime Detoxification of Inhibitory Compounds

Owing to some undesirable degradation products, they cause negatively effect on enzymatic hydrolysis and fermentation steps. The inhibitors including furan derivatives (furfural, 5-hydroxymethyl-furfural (5-HMF)), aliphatic acids (acetic, formic and levulinic acids), and phenolic compounds were released during the degradation of lignocellulosic biomass (García *et al.*, 2011). Furfural and 5-hydroxymethyl furfural (HMF) are two furan derivatives which are formed by the further hydrolysis of the sugars, pentoses and hexoses. These furans are available in relatively high concentration in the hydrolyzates and are known as serious inhibitors to many other microorganisms (Purwadi *et al.*, 2004). The inhibitors, which influence to the enzymatic hydrolysis, consist of formic acid inhibiting cellulases and xylanases, vanillic acid, syringic acid and syringaldehyde inhibiting xylanases (García *et al.*, 2011).

Treatment of lignocellulosic hydrolyzates with alkali has been frequently employed as a detoxification method to improve the fermentability. This process is mainly used to convert furan derivatives into other, less toxic compounds (Purwadi *et al.*, 2004). Detoxification of lignocellulose is hydrolysated by addition of $Ca(OH)_2$, which is considered to be one of the best detoxification methods. $Ca(OH)_2$ adjustment of pH has been reported to result in better fermentability than NaOH adjustment due to the precipitation of toxic compounds (Palmqvist and Hahn-Hägerdal, 2000). After an overliming treatment (pH 10) causing the formation of a large precipitate, the ethanol productivity was further increased. The detoxifying effect of overliming is both to the precipitation of toxic components and the instability of some inhibitors at high pH. In the 1940s, treatment with a reducing agent such as sulphite, was suggested as means to overcome an unfavourable reduction potential in lignocellulosic hydrolysates (Palmqvist and Hahn-Hägerdal, 2000).

Furthermore, many recent studies about the treatment of dilute-acid hydrolysates of spruce with sodium sulphite (Larsson *et al.*, 1999) have been shown to decrease the concentrations of furfural and HMF. A combination of sulphite and overliming has been presented to be the most efficient method to detoxify hemicellulose hydrolysate prior to fermentation. The effect of the combined treatment was probably

decreased due to concentrations of Hibbert's ketones and aldehydes, and the removal of volatile compounds when a heat treatment was employed. However, in order to avoid high pH at elevated temperature, both the sugars and the inhibitors were converted during the overliming process. The degradation of the sugars decreased in mild conditions and rose with increasing pH as well as temperature. A problem associated with alkali detoxification is that not only inhibitors may be affected by the treatment, but also the sugars, which will lead to reduced ethanol yield. The degradation of sugars was extensive when harsh conditions (i.e. long treatment time, high temperature, and high pH) were applied. Xylose was slightly more easily degraded than the other sugars (glucose, mannose, galactose, and arabinose) during the alkali treatments. The goal of the alkali detoxification is to achieve maximum fermentability and minimum sugar degradation at a low cost. The disadvantage of using $Ca(OH)_2$ for alkali detoxification is the formation of a precipitate consisting of $CaSO_4$ (gypsum). If long-term processes are considered the use of $Ca(OH)_2$, which may result in problems with calcium oxalate precipitation and causes clogging of pipework and filters.

2.5 Enzymatic Hydrolysis of Cellulose

Enzymatic hydrolysis of lignocellulosic materials is a very slow process because cellulose hydrolysis is hindered by structural parameters of the substrate, such as lignin and hemicellulose content, surface area, and cellulose crystallinity (Pan *et al.*, 2006). The products of the hydrolysis were normally reduced sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8) and temperature (45 - 50 °C) and does not have a corrosion problem (Sun and Cheng, 2002). Comparison of process conditions and performance of three cellulose hydrolysis processes is given in Table 2.3. Enzymatic hydrolysis is an environmentally friendly alternative that involves using carbohydrate degrading enzymes (cellulases and hemicellulases) to hydrolyze lignocelluloses into fermentable sugars (Keshwani and Cheng, 2009).

Table 2.3 Comparison of process conditions and performance of three hydrolysis process (Keshwani and Cheng, 2009)

	Consumables	**Temperature (°C)**	**Time**	**Glucose yield (%)**	**Available**
Dilute acid	<1 % H_2SO_4	215	3 min	50–70	Now
Concentrated acid	30–70% H_2SO_4	40	2–6 h	90	Now
Enzymatic	Cellulase	50	1.5 days	75→95	Now→2020

Cellulose is typically hydrolyzed by an enzyme called cellulase. These enzymes are produced by several microorganism, commonly by bacteria and fungi. But, most research for commercial cellulase production has focused on fungi because many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, also have a very low growth rate, and required anaerobes growth conditions (Duff and Murray, 1996). Species of fungi that most commonly used are *Trichoderma*, *Aspergillus*, etc. Moreover, mutant strains of *Trichoderma* specie (*T. viride*, *T. reesei*, *T. longgibrachiatum*) have long been considered to be the most productive and powerful destroys of crystalline cellulose (Zhou *et al.*, 2008).

A cellulase dosage of 10 FPU (filter paper units) per gram of biomass is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). Chen *et al.* (2007) investigated effect of cellulase dosage on the enzymatic hydrolysis of dilute acid-treated corncob. Hydrolysis experiment were performed with 100 g/l substrate and different dosages of *T. reesei* ZU-02 cellulase (FPU/g substrate) at pH 4.8 and 50 °C. Result of this study can be concluded that both of reducing sugar concentration and hydrolysis yield had similarly variation trend that is increased sharply with cellulase dosage varying from 10–20 FPU/g substrate, and basically leveled off from 20–30 FPU/g substrate.

One limitation with using cellulase is reduction of rates due to end product (cellubiose and glucose) inhibition. Simultaneous saccharifacation and fermentation (SSF) overcomes this problem by hydrolyzing cellulose and fermenting the hydrolysis product at the same time (Rezaei *et al.*, 2008).

2.6 Acetone-Butanol-Ethanol (ABE) Fermentation

ABE fermentation is a process using bacterial fermentation to produce acetone, n-Butanol, and ethanol from starch, which was used during World War II for making acetone and cordite. This process is anaerobic process related to how yeast ferments sugars to produce ethanol for beer, wine, or fuel. The process produces these solvents in a ratio of 3–6–1, or 3 parts acetone, 6 parts butanol and 1 part ethanol. A strain of bacteria from *Clostridia* class (*Clostridium* Family) is normally used for this process. *Clostridium acetobutylicum* is the most well-known strain, while *Clostridium beijerinckii* has been used for this process with good results.

Microorganisms for ABE fermentation are especially sacchrolytic butyric acid producing clostridia. *Clostridium acetobutylicum* is the most popular and extensively implemented strain, while other butanol produces strain such as *C. aurantibutyricum* produces acetone, isopropanol, and butanol, as well as *C. tetanomorphum* produces equimolar amount of ethanol and butanol, and *C. beijerinckii*, which has a higher selectivity for butanol. When 3:6:1 of total solvent yield 20 g/L is the ratio of ABE produced by *C. acetobutylicum*, *C. berjerinckii* produces up to 3:6:1 with a total solvent yield of 33 g/L (Amrita and Moholkar, 2009). Furthermore, the optimal temperature of *Clostridia* is around 30–40 °C, and between 60–75 °C for thermophilic strains (*C. thermoaceticum*, *C. thermohydrosulfuricum*).

In case of ideal condition, *Clostridia* requires anaerobic gram positive and rod shaped bacteria with the ability to form endospores, and the range of growth pH-value should be 6–7. Moreover, All of strains doesn't require anaerobic conditions; for example, *C. histolyticum* and *C. acetobutylicum* are considered to be aero-tolerant.

In the event of batch conditions, there are two different pathways for *clostridia* cells during solvent production: one for acidonegenesis phase and one for solventogenesis phase (Napoli, 2012). The production of cells, hydrogen, carbon dioxide, acetic acid, and butyric acid during the initial growth phase (acidogenic phase) are operated in the fermentation process of solvent-producing Clostridium strains. While the acids concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenic phase) and acidogenesis cells shift to solventogenesis state with a morphologically change. Especially, endospores from active cells are unable to reproduce themselves, but able to metabolize acids and substrate to produce solvents.

Wang and Blaschek, (2011) studied about the order of important variables on butanol yield is pH > sugar concentration > agitation. A maximum butanol yield of 0.27 g/g-sugar was estimated at the optimal condition of pH 6.7 with a sugar concentration 42.2 g/l, and an agitation rate 48 rpm. Moreover, higher agitation rates caused acetone production, leading to lower butanol fraction in total ABE. While glucose and fructose are more preferable by *C. beijerinckii*.

2.6.1 Fermentation Techniques

Fermentation can be executed as a batch, fed-batch or continuous process. The kinetic properties of microorganisms, type of lignocellulosic hydrolysate, and process economics aspects were informed as the factors for the most suitable process. Batch culture is a closed system, which consists of an initial, limited amount of nutrient. Furthermore, it is inoculated with microorganisms to allow the fermentation. Due to being a very simple method, nothing is required to add during fermentation inoculated with microorganisms to allow the fermentation.

Fed-batch reactors have been widely used in many industrial applications because of the advantages from combination of batch and continuous processes. The major advantage of fed-batch comparing with batch are recognized the ability to increase maximum viable cell concentration, prolong culture lifetime, and allow product accumulation to a higher concentration. This process is appropriate for the maintenance of critical process variables such as temperature, pH, and dissolved oxygen at specific levels through feedback control. In case of

continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the micro-organisms are active. After that the product, which composes of bioethanol, cells, and residual sugar, are taken from the top of the bioreactor. It was found the improvement of the yeast bioethanol fermentation process involved operating the fermenters in a continuous mode comparing to a conventional batch mode and thereby increasing the productivity about threefold from about 2 to 6 g EtOH/l/h. In addition, operating continuously at higher cell densities using cell recycle reactors was another effective means of greatly increasing the productivity. A single-stage continuous stirred-tank reactor (CSTR) with cell recycle operating at high biomass loading of 50–80 g yeast/l produces 30–40 g EtOH/l/h bioethanol.

2.6.1.1 Separate Hydrolysis and Fermentation (SHF)

Enzymatic hydrolysis performed separately from fermentation step is well-known as separate hydrolysis and fermentation (SHF). In the SHF configuration, the joint liquid flows from both hydrolysis reactors and enters the glucose fermentation reactor, and then the mixture is distilled for removing the bioethanol to leave the unconverted xylose behind. For the second one, distillation of bioethanol is presented again owing to fermentation of xylose. The advantage of SHF is the performance to complete each step under optimal conditions; for instance, enzymatic hydrolysis at 45–50 °C and fermentation at about 30 °C. On the other hand, the disadvantage of this method is the inhibition of cellulose and b-glucosidase enzymes by glucose released during hydrolysis, which calls for lower solids loadings and higher enzyme loadings to achieve reasonable yields.

2.6.1.2 Simultaneous Saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) is a promising process option for production of bioethanol and biobutanol from lignocellulosic materials. In SSF, cellulases and xylanases convert the carbohydrate polymers to fermentable sugars. These enzymes are commonly known as sensitive to feedback inhibition by the products– glucose, xylose, cellobiose, and other reducing sugar (Jeffries and Jin, 2000). This process has an enhanced rate of hydrolysis, needs lower enzyme loading, results in higher bioethanol yields, and reduces the risk of contamination. Presently, Olofsson *et al.* (2008) have noted that SSF process for wheat straw

hydrolyzate can be expected to give final bioethanol concentrations close to 40 g/l with a yield based on total hexoses and pentoses higher than 70%.

SSF requires compatible fermentation and saccharification conditions, with a similar pH, temperature and optimum substrate concentration. In many cases, the low pH, e.g. lower than 5, and high temperature, e.g. >40 °C, may be favorable for enzymatic hydrolysis, whereas the low pH can surely inhabit the lactic acid production and the high temperature may affect trouble the fungal cell growth (Huang *et al.*, 2005). *Trichoderma reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 55 °C. For *Saccharomyces* cultures SSF are typically controlled at pH 4.5 and 37 °C (Dien *et al.*, 2003). A typical fermentation will take 5–7 days, depending on the accessibility of the cellulose and initial solids loading of the fermentation.

Sun and Cheng, (2002) have described major advantages of SSF include: (1) increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity; (2) lower enzyme requirement; (3) higher product yields; (4) lower requirements for sterile conditions since glucose is removed immediately and bioethanol is produced; (5) shorter process time; and (6) less reactor volume. The major advantage of SSF is that the immediate consumption of sugars by the microorganism produces low sugar concentrations in the fermentor, which significantly reduces enzyme inhibition compared to SHF (Schell *et al.*, 1998).

2.7 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a procedure to design experiments, build models, and evaluate the effects of abundant factors. This technique can achieve the optimum conditions for desirable responses with a limited number of planned analyses. While traditional optimization has some disadvantages about increasing of experimental necessary to conduct the research, which leads to an increase of time and expenses as well as an rising in the consumption of reagents and materials (Bezerra *et al.*, 2008). Response surface methodology is an assembled technique between Mathematics and Statistics, which explains the behavior of a data

set with the objective of making statistical previsions by the fit of a polynomial equation to the experimental data. It can be well applied when a response or a set of responses of interest are influenced by several variables. The objective is to simultaneously optimize the levels of these variables to attain the best system performance (Bezerra *et al.*, 2008).

RSM helps to describe how a given set of input variables over some specified region of interest affects a particular response, and what input values will yield a maximum (or minimum) for a specific response. Initially, RSM was developed with the purpose for determining optimum operation conditions in the chemical industry, but now it is used in a variety of fields and applications, which includes biological, clinical, and social sciences, not only in the physical and engineering sciences (Khuri, 2001).

Central Composite Designs or CCD can be divided into 3 parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a star design in which experimental points are at a distance from its center; and (3) a central point. The full central composite design for optimization of two and three variables are presented by Fig. 2.7(a and b).

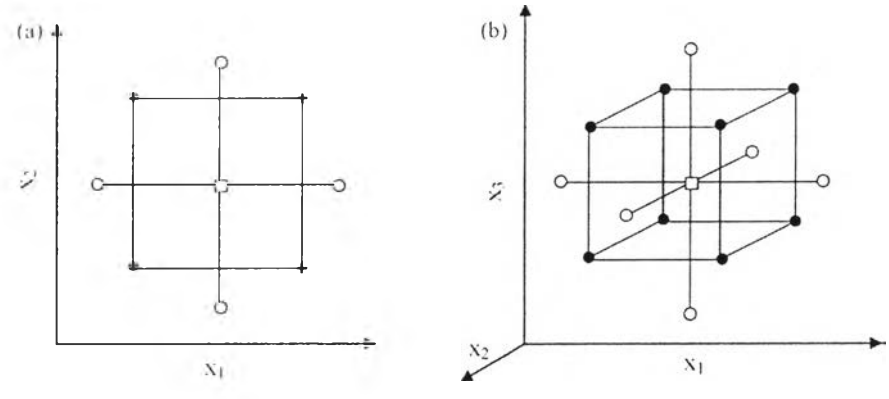

Figure 2.7 Central composite designs for the optimization of: (a) two variables (α = 1.41) and (b) three variables (α = 1.68). (●) Points of factorial design, (○) axial points and (□) central point.

Baboukani *et al.*, (2012) studied the optimization condition of dilute sulfuric acid pretreatment for ethanol production and evaluated the effects of acid concentration, treatment time, and temperature using RSM with central composite designs. In optimization, the desired goals for the responses were chosen to maximize the value of xylose yield and minimize values for glucose yield because it can be desirable that cellulose was suitable access for enzymatic attack after pretreatment. The optimum conditions for sugar recovery were temperature 147 °C, acid concentration 1.53% and retention time for 30 min. Moreover, temperature effect has more significant than acid concentration and treatment time for enhancement of xylose release and cellulose digestion.

Optimization of sugar recovery from rice straw by dilute acid pretreatment was performed for the efficient removal of xylan from rice straw (Kim *et al.*, 2012). The effects of temperature, reaction time, and acid concentration to maximize solubilized xylose concentration and reduce the hydrolysis time for glucose recovery were investigated using RSM. The optimal dilute acid pretreatment process was as follows: temperature 110 °C, reaction time 14.02 min, and acid concentration 1.2%. FT-IR and XRD were carried out in order to prove that the crystalline portion was cellulose. When rice straw was treated with dilute acid, CrI was increased by 35.42-46.68% approximately and clearer and relatively sharp of β-glucosidic bond. These results supported that the crystalline portion was cellulose and could be convertible to the fermentable sugar.

Wang and Blaschek (2011) have studied optimization of butanol production from tropical maize stalk juice. Batch experiments employing central composite design (CCD) and response surface methodology (RSM) were performed to evaluate effects of three factors: pH, initial sugar concentration, and agitation rate on butanol production. A maximum butanol yield of 0.27 g/g-sugar was estimated at the optimum condition of pH 6.7, a sugar concentration 42.2 g/l, and an agitation rate 48 rpm. In order of significant of the variables on butanol yield, the most effect is pH followed by sugar concentration and agitation, respectively. Furthermore, the result shows that higher agitation facilitated acetone production, leading to lower butanol selectivity in total acetone-butanol-ethanol (ABE).

According to all knowledge and literature reviews that previously mentioned, this work will study the two different dilute acids (sulfuric and phosphoric) continuing from the previous research works (Tangmanasakul, 2011 and Satimanont, 2012) with the condition that has given the highest yield of fermentable sugar, 47.11 g/l and 46.14 g/l, respectively. In addition, the effects of overliming on removing inhibitor from prehydrolysate will be investigated. To improve the yield and rate of butanol production, this work will be focused on optimizing the simultaneous saccharification and fermentation process using the non-commercial enzyme, ARR2–7, a generous gift from Siam Victory (Thailand) and microorganism specie of *Clostridium Beijerinckii* TISTR1461. The main variables in this study are temperature, pH, and time.