# CHAPTER II
# BACKGROUND AND LITERATURE REVIEW

## 1. General Description of Butanol

Butanol is a 4–carbon structured alcohol with the molecular formula of $C_4H_9OH$. It was considered as a promising fuel since it has more advantages than ethanol which can also produced from the microorganism as shown in Table 2.1. Compared with other common fuels, butanol also shows good result in terms of energy density and heat of vaporization. Not only as a fuel, butanol is used in the industrial solvent, feedstock for plastic industry, food and cosmetic as an extraction solvent so butanol from petroleum-based is not preferable because it has potential for carcinogen carryover (Formanek *et al.*, 1997). Currently, butanol from the fermentation process gain more interesting because it is environmental friendly, and reduces the petroleum dependence.

**Table 2.1** Properties of common fuels (Anonymous, 2006)

| Fuel | Energy density (MJ/L) | Air-fuel ratio | Specific energy (MJ/kg air) | Heat of vaporization (MJ/kg) | RON | MON |
|---|---|---|---|---|---|---|
| Gasoline and Biogasoline | 32.0 | 14.6 | 2.9 | 0.36 | 91-99 | 81-89 |
| Butanol fuel | 29.2 | 11.1 | 3.2 | 0.43 | 96 | 78 |
| Ethanol Fuel | 19.6 | 9.0 | 3.0 | 0.92 | 107 | 89 |
| Methanol | 16.0 | 6.4 | 3.1 | 1.20 | 106 | 92 |

## 2.2 Acetone-Butanol-Ethanol Fermentation (ABE Fermentation)

### 2.2.1 History

The ABE fermentation is not the modest fashion. The first butyrate forming bacteria was isolated by Louis Pasteur since 1861 named *Vibrion butyrique* and found later that they also produce butanol. They was classified to be anaerobic bacteria meaning that they can not tolerate air or oxygen. In the period of early 20$^{th}$

century, the price of natural rubber raise rapidly because of high demand then the synthetic rubber was discovered for substitution. It was manufactured from butadiene which polymerized from butanol or isoamyl alcohol. At that time, *C. acetobutylicum* was isolated by Chain Weizmann who investigated the possibility of producing butanol by means of a microbial fermentation. This microorganism can tolerate higher acid, utilize sugars from various feedstocks including rice and produce more butanol. This well-known process was patented in 1915 called Weizmann process. Moreover, Weizmann improved the microorganism by applying the heat shock to eliminate weak strain and deoxygenate the culture. This process also recovered riboflavin (vitamin $B_2$) as by-product. In World War II, the demand of acetone is suddenly high since they was used to produce smokeless gunpowder and kept butanol as by-product. The market changed again when World War passed as a low demand in acetone (Jones and Woods, 1986).

The new born in automotive industry from Henry Ford made the indirectly high demand for butanol. Firstly, they required amyl acetate using as solvent for various lacquers. The feedstock for amyl acetate is amyl alcohol ($C_5H_{12}O$, 8 isomers) acquired as by-product of alcoholic fermentation by yeast. Later, there was a prohibition for amyl acetate in USA so the butyl acetate production using butanol as feedstock is used instead. In 1945, there was an impact for acetone and butanol from fermentation process again. The petrochemical process can produce both of them in the lower price and fulfill all of the market. The acetone can be produced from isopropyl alcohol (propylene as feedstock) using copper as a catalyst while butanol can be produced from one of ethylene, ethanol, acetal dehyde, aldol or crotonal dehyde. Many fermentation plants have been shut down because the price of grain and molasses are very high compared with the feedstock from petroleum and another reason is no intention in environmental concern. The opposite phenomena occurred when the oil price increased around 1970. The scientists tried to promote acetone and butanol from fermentation because of non-toxic with environment and lower price. Currently, the obstruct from feedstock price is eliminated by the technology of pretreatment. There are no competition with human food because the feedstock from biomass are viable (Dürre, 2008).

2.2.2 Microorganism

The *C. acetobutylicum* is a gram-positive bacillus mostly found in soil dwelling at the optimal temperature of 10–65 °C. It can break down sugar to produce solventogenic products, saccharolytic. The rod–like cells have movement through flagella if they can grow well also implied the increasing in solvent production due to chemotaxis. This spore-forming bacteria required anaerobic conditions to grow in its vegetative state but it can survive in the aerobic condition for several hours later it will form spore and living for years. For the spores, at the right condition, they can continually grow. The solventogenic products made from this culture is mainly acetone, butanol, and ethanol at the mass ratio of 3:6:1, while butyrate and acetate are considered to be by-products appearing in exponential growth phase when nitrogen fixation occurs. In the collection of relative strains, there are no standards for the classification of these organisms. The most commonly studied strain is *C. acetobutylicum* ATCC824 isolated from soil in Connecticut garden in 1924. For the other species, *C. aurantibutyricum* can produce isopropanol, acetate, and butanol. *C. tatanomorphun* can generate only butanol and ethanol in the equimolar ratio. Currently, in addition to *C. acetobutylicum,* the *C. beijerinckii* is more interested in the study because it is believed to be higher tolerance to the toxic solvent and produce more solvent. By the way, both *C. acetobutylicum and C. beijerinckii* are considered to be very similar species. The differences of these species, for example, are the structural organism that affect the degeneration, the loss of ability to produce solvents, and the formation of spore after repeated subculture or continuous cultivation. The *C. acetobutylicum* ATCC824 has a 210-kb plasmid encoding several solventogenic genes meaning the degeneration is caused by losing this plasmid while *C. beijerinckii* NCIMB8052 has a single circular chromosome but no plasmid thus the degeneration occurred through genetic alteration to the chromosome (Lee *et al.*, 2008).

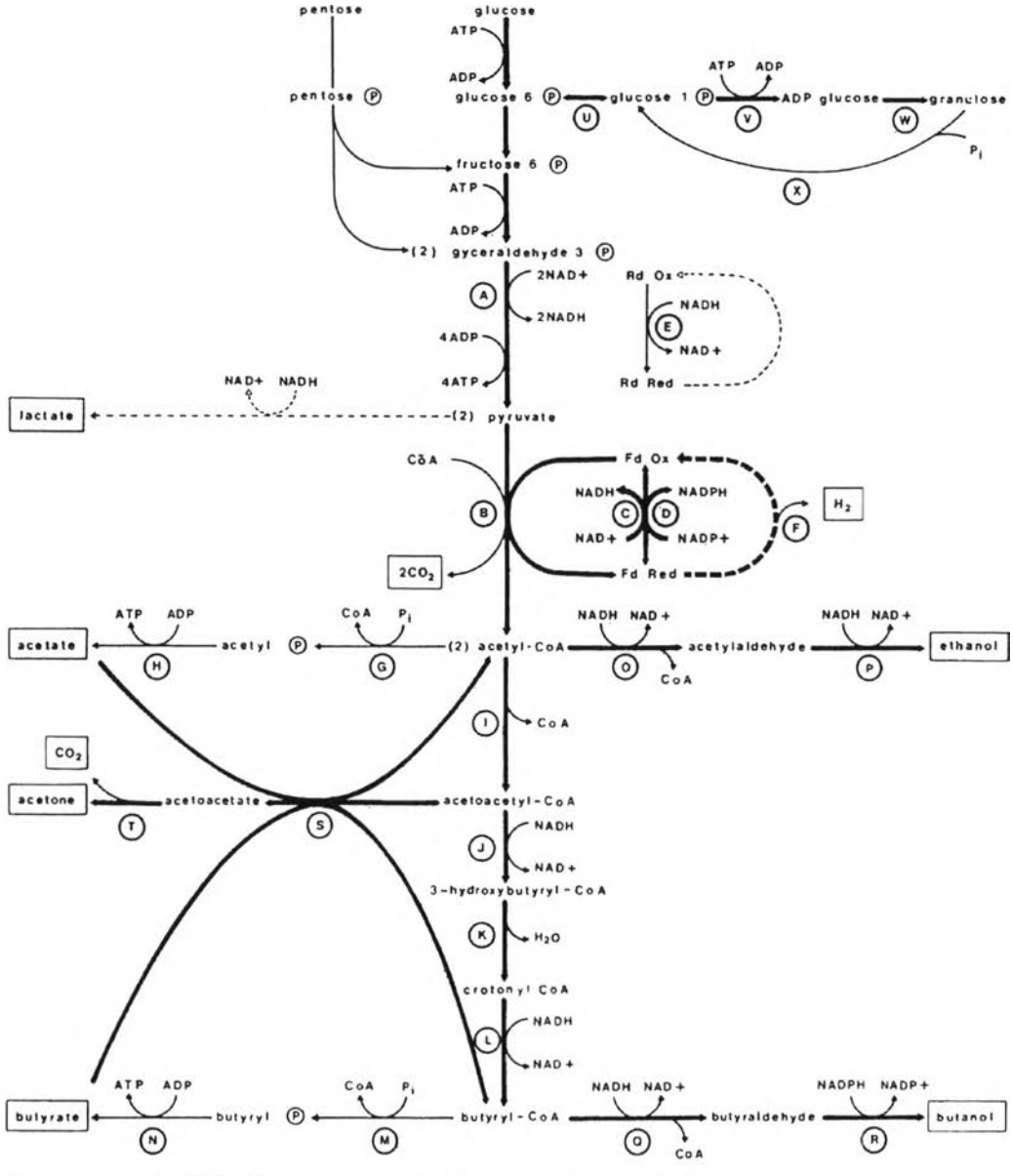
At present, *C. beijerinckii* BA101 is considered to be the hyperamylotic, solvent-hyperproducing mutant from the parental strain of NCIMB8052. Compared with NCIMB8052, it can produce 69% more total solvent and can tolerate butanol up to 23 g/L (typically about 13 g/L). This strain, BA101, is generated from NCIMB8052 by using N-methyl-N'-nitro-N-nitrosoguanidine with selective enrichment on the glucose analog 2-deoxyglucose and it is promised to be newly strain for

ABE fermentation (Annous and Blaschek, 1991). There is an assumption that butanol is produced from the reduction of butyric acid; therefore, the fermentation with co-cultures that supplying a large amount of butyric acid is studied (Formanek *et al.*, 1997).

As it was mentioned above that the commonly strains used in the study of ABE fermentation were *C. acetobutylicum* and *C. beijerinckii*. Then there are many reported condition concerning with the supplementary in the production medium and various type of raw materials. To make the comparative study reasonable, these two strains were firstly chosen as a representative to its specie. Both of . *C. acetobutylicum* and *C. beijerinckii* were maintained as a culture collection at Thailand Institute of Scientific and Technological Research (TISTR) as the strain of TISTR 1462 and TISTR 1461 which were similar to *C. acetobutylicum* Dsm 4685 and *C. beijerinckii* Dsm 6422, respectively. They were originally isolated from different country and have no study in the ABE fermentation using P2 production medium which can modify to enhance the solventogenic products. Then the criteria of strain selection was firstly rely on the condition involving with the strains used in the study of enhancement by the addition of supplementary to the medium. For instances, the addition of ammonium acetate used in the study of solventogenic enhancement was performed at 37 °C (Gu *et al.*, 2009) then this temperature was considered as a criteria for strain selection since other temperature may alter the solubility of ammonium acetate.

### 2.2.3 Pathway

Both *C. acetobutylicum* and *C. beijerinckii*, they can degrade sugar in the same manner as the other Clostridia but the characteristic of the ABE fermentation pathway is that the occurring of 2 phases, acidogenesis and solventogenesis. In addition to sugar, they can also use starch and xylan mediated with α-amylase and partially endoxylanases for growth but cannot directly utilize cellulose. The main step of ABE fermentation is the conversion of carbohydrates to acids (acetate and butyrate), solvents (acetone, ethanol and butanol), and gas ($CO_2$ and $H_2$). The microorganisms can consume both hexose and pentose sugar that different from ethanol production required only for hexose sugar (Kumar and Gayen, 2011). The biochemi-




cal pathway of ABE fermentation is shown in Figure 2.1.

**Figure 2.1** Metabolic pathways of *C. acetobutylicum* (Jones and Woods, 1986).

For 1 mole of hexose sugar, they are metabolized via glycolysis pathway (Embden-Mayerhof pathway) and end up with 2 mole of pyruvate, 2 mole of ATP (adenosine triphosphate) and 2 mole of NADH (reduced nicotinamide dinucleotide). But not for pentose sugar, they have to flow through pentose phosphate pathway (PPP) before enter the glycolytic pathway. However, they also lead to both acid and solvent production.

In the batch of ABE fermentation, *C. acetobutylicum* produces $H_2$, $CO_2$, and acids at the initial of exponential growth phase. The accumulation of acids makes pH going down called acidogenic. In this acid producing pathway, acetic and butyric acids are produced from 3 key intermediates (acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA) that generated initially from pyruvate. The metabolism will shift to the solventogenic phase when the concentration of butyric acid reach 2 g/L and pH lower than 5 (Jones and Woods, 1986).

The metabolic shift can be described from pathway, firstly in the exponential growth phase, they tend to produce only acid products, butyrate and acetate because each produces ATP which required for cell growth. In these 2 acid products, butyrate consumed NADH that preferable for resolve the issue of redox equilibrium then the butyrate is more produced than acetate. Later in the stationary phase, most of butyrate and acetate are converted to butanol and ethanol respectively called solventogenic phase known as the mechanism to prevent the rapidly going down pH (Jones and Woods, 1986; Maddox *et al.*, 2000).

The detailed in the molecular biology of *C. acetobutylicum* was described by Nölling *et al.* (2001). The megaplasmid pSOL1 of 192 kb was discovered as its lead to the degeneration of the strain (Cornillot *et al.*, 1997). The diffrence between some strains of *C. acetobutylicum* and *C. beijerinckii* was also identified by the degeneration from pSOL1 pasmid. The enzymes concern with the solventogenic reactions are encoded in five different operons (Table 2.2).

**Table 2.2** Regulation of *C. acetobutylicum* genes required for solventogenesis (Dürre, 2008)

| Operon | Relative promoter strength | Upstream OA boxes | Regulatory mechanisms[a] |
|---|---|---|---|
| *adc* | 100 | 2 + 1 reverse | Induction of transcription Modification of gene product |
| *sol (orfL-adhE-ctfA-ctfB)* | 1.2 | 1 reverse | Induction of transcription mRNA processing |
| *bdhB* | 77 | 1 | Induction of transcription |
| *bdhA* | 4.5 | 2 | None, constitutive expression under these conditions |
| *adhE2* | ND[b] | 1 | Induction of transcription[c] |

[a]Switch from acidogenesis to solventogenesis in continuous culture.
[b]Not determined.
[c]Under "alcohologenic" conditions (e.g., substrate combination glucose/glycerol).

### 2.2.4 Nutrient Limitation

Nutrient limitation has highly affect to the solvent production. There are many studies concerning with carbon, nitrogen, phosphate, and sulfate limitation in both batch and continuous culture. In the conclusion, the excess of carbon sources are essential for cell growth and shift their metabolism to solvent production. In batch cultures, present with glucose concentration below 7 g/L, there are only acid obtained in the broth meaning that cells cannot grow and produce acid much more to reach acid crash. In contrast, for continuous cultures, the feed rate of glucose is more important than its concentration (Jones and Woods, 1986; Maddox *et al.*, 2000).

The effect of nitrogen limitation on the production of solvents is also important as carbon source. At low level of ammonium (commonly as a nitrogen source for cultures) in the medium cause the decrease in produced biomass and utilized glucose. It is the same reason as carbon limitation that cells cannot reach the threshold concentrations of acid then they do not shift to solventogenic (Jones and Woods, 1986).

### 2.2.5 Temperature and Oxygen

The most temperature recommended for Clostridia is 37 °C but 30 °C is also proper for some strains (Ennis and Maddox, 1985). Temperature can affect yield, solvent ratio, and production rate. There were reported that fermentation time decreased as the temperature was increased and also lower acetone production. That is why solvent ratio was changed. Moreover, temperature has effect on butanol toxicity (Knoshaug and Zhang, 2008).

Oxygen definitely affect this anaerobic microorganisms. For short period, exposure of oxygen can not diminish cultures but high oxygen concentration made low glucose consumption, growth rate, and block DNA, RNA synthesis. Under aerobic condition, there were significantly decrease in butyrate production (but not for acetate) then, from pathway, the intracellular ATPs decline further. When the condition change back to anaerobic, cultures metabolism and growth can resume their function. Thus, there is considered to be reversible effect (Jones and Woods, 1986).

### 2.2.6 Limitation of ABE Fermentation

Although the ABE fermentation plants can produce butanol at large volume for several decades but there are some limitations made them no economic feasible compared with butanol from petrochemical process.

The process can not use biomass directly as carbon substrates thus the pretreatment step required to hydrolyze them to monosaccharide viable for microorganism adding cost to raw material. Other than that the fermentation process needed to be run under sterile condition to prevent contamination.

One of the limitations that made cultures produce low solvent yield is solvent toxicity. There is inhibitory level of solvent around 20 g/L. Butanol is the most toxic. Cell growth and solvent production fail when butanol concentration reaches about 13 g/L in the industrial fermentation process. There are studies that adding butanol at the concentration of 7–13 g/L to the medium. The results show that culture were cease about 50 % in growth when using hexose sugars as substrate and totally inhibited at the concentration of 12–16 g/L. The threshold value for butanol toxicity that affect growth of culture is 4–4.8 g/L. In contrast, the butanol at the con-

centration not over than 8 g/L can enhance cultures grown in xylose (Moreira *et al.*, 1981).

For further genetic engineering, Knoshaug *et al.* (2008) screened 24 different microorganisms other than clostridium strains and found that they can tolerate butanol at the concentration of 1–2 %. Strains of *Escherichia coli*, *Zymomonas mobilis*, non-Saccharomyces yeasts and *Saccharomyces cerevisiae* can not tolerate 2 % butanol in accordance with Clostridium strains resulting in 60 % growth rate decrease. The promising one is two strains of *Lactobacillus* (*L. delbrueckii and Lactobacillus brevis*). They can survive in 2 % butanol with 80 % growth compared with no butanol addition medium.

Not only butanol that inhibit growth of microorganisms but also for other aliphatic alcohols. Izard and Goma (1989) studied the effect of various alcoholic supplements divided into 2 groups: linear (methanol, ethanol, propanol, butanol, hexanol and octanol) and cyclic (Benzyl alcohol Phenethyl) alcohols. There has directly affected on membrane fluidity. The long chain alcohol fills many gaps in cytoplasmic membrane lowering fluidity different from cyclic chain that leave the gaps. They also suggest that membrane is the site of inhibitory interactions.

From the study of butanol effect to *C. acetobutylicum* (Bowles and Ellefson, 1985), it was found that butanol had numerous effects on the ability of cells to maintain its internal pH and to abolish the membrane pH gradient. Furthermore, the toxicity of butanol also hinders the uptaking sugars and amino acids. Thus, to prevent the butanol toxicity, there are some techniques recovering solvents from fermentation broth reducing the product concentration such as membrane and gas stripping etc.

Another limitation is strain degeneration especially for industrial fermentation. The strain degeneration is the phenomena that strains lose the ability to produce solventogenic products and to form spore when repeated subculture or continuous cultivation (Chen and Blaschek, 1999b). The two clostridia strains, *C. acetobutylicum* and *C. beijerinckii*, can also be degenerated in this situation with the different reasons. For *C. acetobutylicum*, in the degeneration process, they lost 210-kb plasmid (pSOL1) encoding solventogenic genes (ctfA, ctfB, and adhE/aad) whereas *C. beijerinckii*, having no pSOL1 plasmid (solventogenic genes encoded on the sin-

gle circular chromosome), has genetic alternations to the chromosome which contains *sol* operon encoding CoA transferase, aldehyde dehydrogenase and acetoacetate decarboxylase.

Lee and coworkers (2008) suggest that there is a way to prevent the degeneration and also enhance the butanol production by adding acetate and butyrate to the medium. Over than that, the addition of these compounds resulted in a changing in a product ratio. For instance, adding 36 mM of sodium acetate can enhance the overall solvent production and change the ratio of acetone:butanol from commonly at 1:2 to 1:3. This advantage is affect to the cost of separation of fermentation broth because the doubling of butanol concentration from 1% to 2% can lower 62% cost of distillation (Philips and Humphrey, 1983).

## 2.3 Alternative Fermentation Substrates

One hindrance for fermentation process that cannot compete the butanol from petroleum process is cost of substrate. Many fermentation plants were shut down due to the high cost of molasses. Thus, the possible solution to lower cost of production is efficiency of microorganism to utilize various substrates especially when substrates from agricultural parts are over produced. Another thing is lower the nutrient added in the fermentation broth because substrate contained all of carbon sources, nitrogen sources and vitamins, minerals without added supplements can lower cost of production.

Besides molasses that have high cost, lignocellulose is promising to be the substrate for almost fermentation process required sugar even there is necessary to pretreatment first. Because lignocellulose has high productivity and less requirement of water and fertilizer. Lignocellulose called from the 3 major components provided structure for plants: cellulose (38–50 %), lignin (15–30 %), and hemicellulose (23–32%).

Cellulose is a linear polymer of glucose linked by β-1,4 bonds similarly to starch that has α-1,4 bonds. Their bonds different in enzyme activity. For same enzyme loading, amylase hydrolyzes starch about 100 times faster than cellulase hydro-

lyzes cellulose because of cellulose's strength structure is difficult to be digested. Lignin acts as glue in plant's structure. It is a polymer of phenyl propane units linked by ether bonds. Hemicellulose can be hydrolyzed to obtain five-carbon sugars (mostly xylose). It is chemically bonded to lignin and serves as an interface between lignin and cellulose.

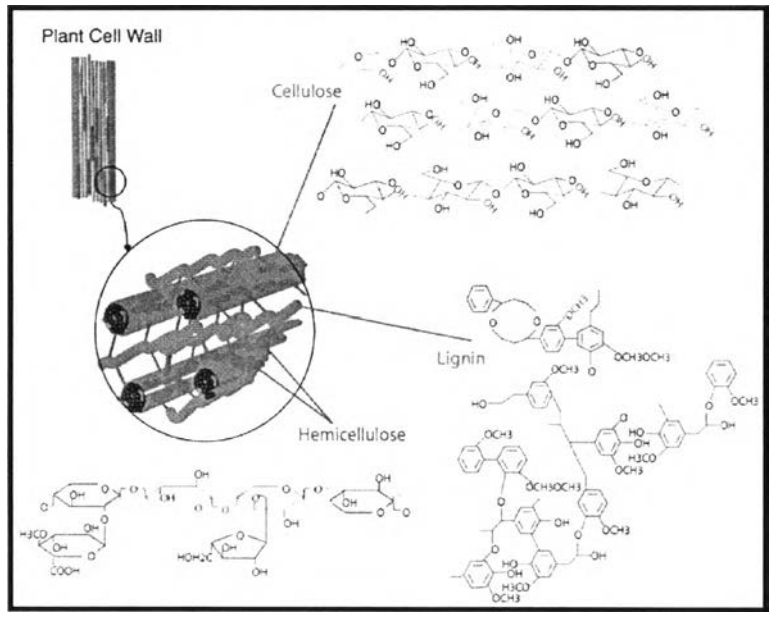



**Figure 2.2** Lignocellulose consists of cellulose, lignin, and hemicellulose (Holtzapple, 2003).

### 2.3.1 Pretreatment

Although lignocellulosic biomass has lower cost and high productivity, but in term of butanol production, microorganisms cannot utilize them directly. Lignocellulosic biomass have to be digested firstly to monosaccharaides. There are some factors affecting the digestibility of enzyme. For instances, the content of lignin, hemicellulose, and acetyl, all of them hinder enzyme accessing to cellulose and hemicellulose. They are all necessary reducing by chemical pretreatment to enhance cellulose digestibility. Other than that, there are the hinder effect of cellulose crystallinity, degree of polymerization, surface area, and pore volume of lignocellulosic biomass.

Thus, the pretreatment step is the requirement making lignocellulosic as substrate for microorganisms or enhance the digestibility of enzyme. They aim to:

- removing or altering lignin
- removing hemicellulose
- decrystallizing cellulose
- removing acetyl groups from hemicellulose
- reducing the degree of polymerization in cellulose
- expanding the structure to increase pore volume and internal surface area.

The pretreatment step can be done by chemical, physical, and biological techniques. Although there have many techniques that can solve these problems but their operating cost are too high so they have to focus only techniques that require minimal energy and minimize capital and operating costs.

From many techniques, the practical one that can be done in lab scale in butanol production is dilute sulfuric acid pretreatment with enzyme. Qureshi and coworkers (2007) produce butanol from hydrolyzed corn fiber using *C. beijerinckii* BA101. In this work, the pretreatment with sulfuric acid is the prerequisite to obtain high sugar yield from corn fiber. However, the controlled experiment showed that the resin XAD-4 is important factor to remove inhibitor formed in acid pretreatment because in the absence of XAD-4, even the similar sugar concentration, the ABE concentration is lower than controlled experiment about 10 times. With the resin, inhibitor remover. the ABE concentration is 6 times higher. There are many studies for various substrate, pretreatment and strains listed in Table 2.3

**Table 2.3** Production of butanol from wood or agricultural residue hydrolysates (Qureshi, 2008)

| Substrate | Hydrolysis method | Treatement to remove inhibitors | Culture | ABE produced (g/L) | Reference |
|---|---|---|---|---|---|
| Pine | SO2-catalysed prehydrolysis + enzyme hydrolysed | None | *C. acetobutylicum* P262 | 17.6 | Parekh *et al.* (1988) |
| Aspen | Above | None | Above | 20.1-24.6 | Parekh *et al.* (1988) |
| Bagasse | Alkali pretreated + enzyme hydrolysed | Ammonium sulfate + activated carbon | *C. roper-butylacetonicum* ATCC 27022 | 18.1 | Soni *et al.* (1982) |
| Rice straw | Above | Above | Above | 13.0 | Soni *et al.* (1982) |
| Wheat straw | Above | None | *C. acetobutylicum* IFP 921 | 17.7 | Marchal *et al.* (1984) |
| Corn stover | SO2-catalysed prehydrolysis + enzyme hydrolysed | None | *C. acetobutylicum* P262 | 25.8 | Parekh *et al.* (1988) |
| Corn fiber | Dilute sulfuric acid | XAD-4 resin | *C. beijerinckii* BA101 | 9.3 | Qureshi *et al.* (2008) |

### *2.3.1.1 Acid Pretreatment and Detoxification Process*

Among the chemical pretreatment, acid pretreatment is the most popular method since it requires low cost chemical and effectiveness. The aim of acid pretreatment is to solubilize the hemicellulose and remain lignin to exposure the cellulose then left porosity and roughness proper for further enzymatic hydrolysis. Moreover, the solubilized hemicellulose was broken down into monosaccharide (Zheng and Pan, 2009), xylose (5 carbons sugar), which can be supplied in the ABE fermentation. Nevertheless, in the presence of dilute acid at high temperature, other than monomeric sugar, inhibitors were formed from the deacetylation of xylan such as acetic, ferulic, glucuronic, ρ-coumaric acids, furan dehydration products including furfural and hydroxymethylfurfural (HMF), aliphatic acids and assorted phenolic compounds from lignin (Martinez *et al.*, 2000), as shown in Figure 2.3. Then the process of detoxification was necessary to apply prior the ABE fermentation. Many techniques were studied expecting to eliminate the inhibitors without reducing amount of sugars in hydrolysate or other nutrients. The nanofiltration (Sun and Liu, 2010) and XAD-4 resin (Qureshi *et al.*, 2008) was a promising method since they were effective in reducing inhibitors but consumed high cost of operation and

maintenance. The overliming process is one of inhibitor removal method required low cost of lime and simply to operate.

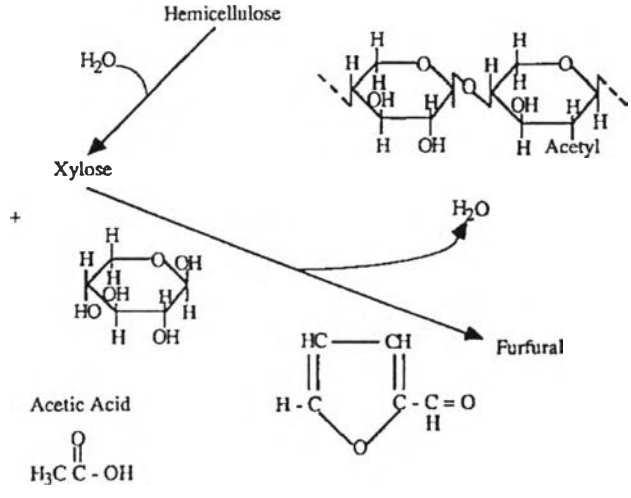



**Figure 2.3** Acid-catalyzed pathway for the hydrolysis of hemicellulose to xylose and the degradation of xylose to furfural (Weil *et al.*, 2002).

There are many studied in various type of acids using in the pretreatment of biomass including dilute sulfuric acid (Canilha *et al.*, 2011; Redding *et al.*, 2010; Schell *et al.*, 2003), dilute nitric acid (Zhang *et al.*, 2011), and dilute phosphoric acid (Gómez *et al.*, 2009; Um and Karim, 2003; Vázquez *et al.*, 2007) in a varied type of biomass but the most effective acid in term of xylose yield and enhancement of enzymatic digestion was sulfuric acid. Though, drawbacks of sulfuric pretreatment were necessary to consider as it requires high grade of stainless steel reactor to prevent the corrosion of sulfuric. Moreover, in a neutralization step or overliming process prior the fermentation, abundant salts form and have to further remove by filtration.

*2.3.1.2 Base Pretreatment*

The advantage of alkaline pretreatment is the delignification with small amount of solubilized hemicellulose. Like acid pretreatment, alkaline pre-

treatment decrease degree of polymerization, crystallinity, and increase biomass surface area (Zheng and Pan, 2009). With this method, lignin, which acts as glue in the biomass structure, is removed in the saponification between the intermolecular ester bonds then the attack of enzyme may take place easily. The studied chemicals of alkaline pretreatment included sodium hydroxide (Kang *et al.*, 2011; Wang *et al.*, 2010), lime (Saha and Cotta, 2010), and alkaline peroxide (Saha and Cotta, 2006). Compared with other pretreatments, it usually requires low temperature and long time along the process. The major disadvantage is the conversion of alkali into irrecoverable salts, which can be removed with the costly dialysis process or else it may inhibit the growth of microbes.

### 2.3.2 Enzymatic Hydrolysis

This process is the most important step to extract fermentable sugars from biomass. Compared with the pretreatment process, most sugars yielded in the enzymatic hydrolysis. Nevertheless, the enhancement of the structure of biomass by the chemical pretreatment is necessary since it increase the digestibility significantly. Enzyme using in this step commonly called Cellulase as it cleaves internal bonds in the cellulose (1,4-β-D-glycosidic linkages) and breaks down into monomeric sugars. It mostly produced by fungi, bacteria, protozoans, and also termites. Sorted on the type of reaction, cellulase enzyme is categorized into 5 types (Anonymous, 2003):

- Endocellulase hydrolyses internal bonds at non-terminal regions yielding oligosaccharides.
- Exocellulase further hydrolyses oligosaccharides produced from endocellulase, resulting in disaccharides (such as cellobiose) or tetrasaccharides.
- Cellobiase or β-glucosidase hydrolyses the exocellulase product into monosaccharides.
- Oxidative cellulases depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase (acceptor).
- Cellulose phosphorylases depolymerize cellulose like oxidative cellulases but using phosphates instead of water.

The reactions of endocellulase, exocellulase, and cellobiase are shown in Figure 2.4. The enzymatic hydrolysis of cellulose to monosaccharides requires a combination of enzyme. However, the other enzyme which often included in cellulase enzyme is Xylanase. It sometimes was added to completely hydrolyse biomass as they can cleave the linear polysaccharide β-1,4-xylan into xylose.

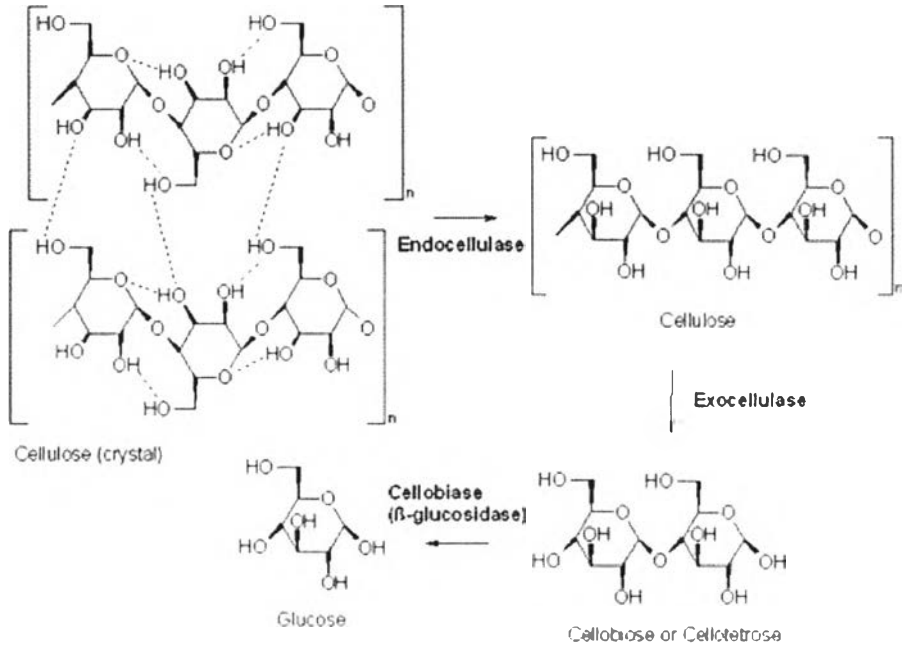



**Figure 2.4** Three consequently reaction of cellulase enzyme (Anonymous, 2003).

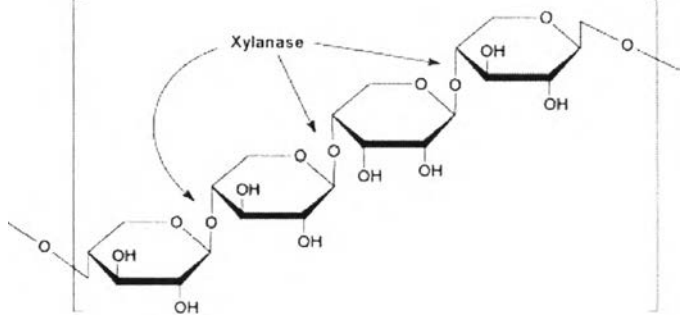



**Figure 2.5** Xylanase hydrolyse Polymer of β-(1,4)-D-xylopyranosyl units (Sigma-

Aldrich, 2013).

## 2.4 Solventogenic Product Enhancement

The regulation of supplementary uptake by Clostridia strain to produce ABE has been studied focusing on chemical that involve in the solvent producing pathway such as acetate (Chen and Blaschek, 1999a) and butyrate (Bahl *et al.*, 1982; Lee *et al.*, 2008). They show that different strain produced different ABE at the optimum condition of acetate (Chen and Blaschek, 1999a, b). It has to be noted that the supplementary of acetate and butyrate can also prevent strain degeneration after subcultures over than 10 times (Chen and Blaschek, 1999a). Other than that, *Clostridium acetobutylicum* EA 2018 can enhance the solventogenic products by ammonium acetate (Gu *et al.*, 2009) instead of solely acetate substance when using cassava as a substrate for ABE fermenation. This result was confirmed by reverse transcription–PCR analysis which show the over expression of solventogenic genes in both acidogenesis and solventogenesis phase after the addition of ammonium acetate compared to the culture that had no addition of ammonium acetate. All substances believed to enhance the solventogenic products since, from the pathway in Figure 2.1, they might shift the metabolic pathway toward acetone and butanol production. Then the effect of the supplementary uptake has to be considered precisely based on each strain and raw materials.

Not only the supplementary in the production medium has an effect to the ABE concentration but also the sugar composition contained in the preculture medium. There were reports from Ounine *et al.* (1985), Jeffries and Sreenath (1988), and Kanouni *et al.* (1998) indicated that the xylose pregrown–cell of *C. acetobutylicum* can utilize xylose contained in the production medium efficiently compared to the glucose pregrown–cell. This is important due to the main component in the substrate obtained from lignocellulosic biomass is xylose.