EFFECTS OF PRETREATMENT AND ENZYMATIC HYDROLYSIS ON OIL PALM FROND FOR USE AS YEAST CULTIVATION MEDIUM

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microorganism to start bioconversion process. Basically, there are two major hydrolysis processes: Acid hydrolysis and enzymatic hydrolysis.

2.4.1 Acid hydrolysis

Acid hydrolysis can be performed by dilute-acid and concentrated acid (Sun and Cheng, 2002; Wenzl, 1970). Normally, mineral acids such as sulfuric acid or hydrochloric acid are used in acid hydrolysis. Concentration of acid used in dilute-acid hydrolysis is usually not more than 2% at temperature between 120 – 200 ºC (Grethlein and Converse, 1991). Whereas, concentrated-acid hydrolysis performed by high concentration acid, 35-60% at moderate temperature, 20-100 ºC (Wenzl, 1970).

The advantage of acid hydrolysis is time consumed is much lower (10 minutes to 6 hours), as compared to enzymatic hydrolysis time is counted in days (Goldstein, 1983 and Wenzl, 1970). However, acid hydrolysis is hampered by non-selectivity and by-products formation from sugar decomposition (Fan et al., 1982).

During acid hydrolysis, hemicelluloses are degraded to xylose, mannose, acetic acid, galactose and glucose; cellulose is hydrolyzed to glucose. At high temperature and pressure xylose is further degraded to furfural (Dunlop, 1948). Similarly, 5-hydroxymethyl furfural (HMF) is formed from hexose degradation, and HMF may be further degraded to form levulinic acid (Ulbricht et al., 1984). During acid hydrolysis, partial of lignin will be degraded to phenolic compounds (Bardet et al., 1985)

Those by-products are fermentation inhibitors. When studying ethanol production by yeast *Pichia stipitis*, Robert et al. (1991) observed that furfural

concentration lower than 0.5 g/l had a positive effect on cell growth, whereas concentration above 2.0 g/l inhibited cell growth almost completely. According to Delgenes et al. (1996), *P.stipiitis* growth was reduced by 43%, 70% and 100% when HMF concentration in the medium was 0.5, 0.75 and 1.5 g/l respectively.

In the lignocellulose hydrolysates, the concentration of sugars as well as the concentration of hydrolysis by-product is depended on hydrolysis conditions (Clausen and Gaddy, 1988). The hydrolysis temperature, time, and acid concentration influence the generation of inhibitor. To combine the residence time and temperature during hydrolysis into single reaction ordinate, Overend and Chornet (1987) had defined a severity factor according to following equation:

$$\text{Log } R_o = t \, e^{(T-100)/\,14.75}$$

Where t is residence time and T is hydrolysis temperature.

Chum et al., (1990) introduced a third parameter, environmental pH (reflecting the amount of acids), into above equation to describe the combined severity (CS):

$$CS = \text{Log } R_o - pH$$

The influence of CS during diluted-acid hydrolysis of spruce on the concentration of fermentable sugars and sugar degradation products has been studied by Larsoon et al. (1998), by varying parameter T (150-240°C), t (1-30 min) and sulphuric acid concentration (0.5-4.4% w/w dry matter) . According to Larsoon et al (1998), when the CS of hydrolysis condition increased, the yield of fermentable sugar increased to a maximum between CS 2.0-2.7 for mannose, 3.0-3.4 for glucose above which it decreased. The decrease in

monosaccharides coincided with maximum concentration of furfural and 5-HMF. With further increase in CS, the concentration of furfural and 5-HMF decreased while the formation of formic acid and levulinic acid increased.

Generally, provided that the inhibitors are identified, the fermentibility of hydrolysate produced from acid hydrolysis can be improved in several ways as suggested by Palmqvist and Hahn-Hägerdal (1999). Firstly, the formation of inhibitors can be minimized through optimization of the pretreatment and hydrolysis conditions. Secondly, prediction of fermentability based on analysis of the hydrolysates will be possible, and thirdly, specific detoxification methods can be developed for efficient removal of inhibitors prior to fermentation. Palmqvist and Hahn-Hägerdal (1999) and Mussatto and Robert (2004) have suggested inhibitory mechanisms and detoxification methods.

Besides the problem of inhibitory of by-product, Sun and Cheng (2002), has conclude in their studies on hydrolysis of lignocellulose materials, that concentrated acid including sulfuric acid and hydrochloric acid are toxic, corrosive, and hazardous, requiring corrosion-resistant reactors. Acids also must be recovered after hydrolysis to make the process economically feasible.

2.4.2 Enzymatic hydrolysis

Cellulose is the major carbon source which can be liberated into glucose, then converted to bio-fuel, chemical bulks and etc through bioconversion process. In this study, cellulose is used as target of hydrolysis although hemicelluloses also can be converted to xylitol and furfural, because glucose is the monomer sugar which can be fermented by most of organism, and also it's high contain in OPF compare to others.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Beguin and Aubert, 1994). The result of hydrolysis will be production of reducing sugar, mainly glucose. Because of high specific, enzymatic hydrolysis does not have sugar decomposition issue and will produce higher yield of sugar compared to acid hydrolysis (Spano, 1977, Goldstein, 1983). Utility cost of enzymatic hydrolysis also is lower compared to acid because enzymatic hydrolysis is usually conducted at mild conditions (pH 4.8, and temperature 45 – 50 ℃) and does not require corrosion-resistant reactor (Duff and Murray, 1996).

Enzyme cellulase can be produce by both of bacteria and fungi for hydrolysis of lignocellulosic materials. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora* and *Streptomyces* can produce cellulase (Bisaria, 1991). Although many cellulolytic bacteria, particularly the anaerobes e.g. *Clostridium thermocellum* and *Becteriodes cellulosolvens* produce cellulase with high specific activity, they do not produce high enzyme yield. Because the anaerobes have very low growth rate and require anaerobic conditions (Duff and Murray, 1996). Most of the research for commercial cellulase production has focus on fungi.

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Among those fungi, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1976)

2.4.2.1 Cellulase

Cellulases are usually a mixture of several enzymes. There are three major groups of cellulases: (1) endoglucanase, (2) exoglucanase or cellobiohydrolase (CBH) and (3) β-glucosidase.

Endoglucanase or endo-1,4-D-glucanohydrolase (EC 3.2.1.4) attacks regions of low crystallinity in cellulose, creating free chain-ends. Endoglucanase also attacks cellulose chains randomly, producing glucose, cellobiose, and cellotriose (Bisaria and Ghose, 1981, Ladish et al., 1983). This will also reduce DP of cellulose chain (Chang, 1974). Exoglucanase or cellobiohydrolase (CBH, 1, 2-β-D glucan cellobiohydrolase, EC 3.2.1.91) cleaves 2-4 units from the ends of the exposed chains produced by endoglucanase, resulting in the tetrasacchrides and disaccharides such as cellobiose. β-glucosidase or cellobiase (EC 3.2.1.21) is use to catalyze break down of cellobiose to glucose (Coughlan and Ljungdahl, 1998).

Although cellulase isolation techniques have not been fully developed, the hypothesis depicted in **Figure 2.7** is now accepted. According to this hypothesis, in a synergistic sequence of events, endoglucanase acts randomly on the cellulose chain, while exoglucanase acts on exposed chain ends by splitting off cellobiose or glucose. Cellobiose is subsequently hydrolysed by β - glucosidase to glucose.

2.4.2.2 Hemicellulase

Besides cellulose, hemicellulose is also main carbon source for fermentation process, especially during production of xylitol. Xylan and glucomannan are major component of hemicellulose (as discussed in 2.3.1.3).
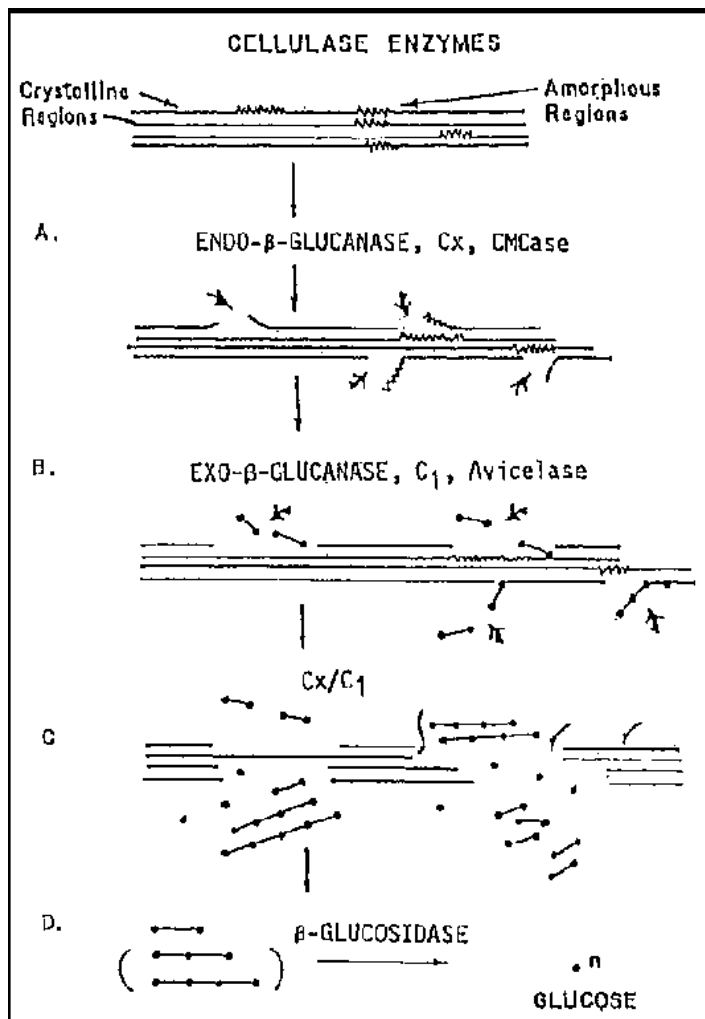
Figure 2.7 Schematic representations of sequential stages in enzymatic hydrolysis of cellulose (Anon, 2008b)

The enzymes used to hydrolysis hemicelluloses are Endoxylanase (EC 3.2.1.8) and Endomannanases (EC 3.2.1.78) which attack the main chain of xylan and glucomannans respectively. Oligomeric compounds produced are then further hydrolyzed by β-xylosidase (EC 3.2.1.37), β-mannosidase (EC 3.1.1.25) and β-glucosidase (EC 3.2.1.21). The side groups that still attached to oligosaccharides can be removed by α-glucuronidase (EC 3.2.1.139), α-arabinosidase (EC 3.2.1.55) and α-D-glalactosidase (EC 3.2.1.22). Acetyl

substituents bond to hemicelluloses can be removed by esterase (EC 3.1.1.72). (Viikari et al., 2001)

2.4.2.3   Factors limiting enzymatic hydrolysis

Generally, hydrolysis of lignocellulose material is affected by physiochemical properties / structure of substrate (e.g. crystallinity of cellulose, polymerization degree of cellulose, particle size, and presence of lignin and hemicelluloses) and hydrolysis conditions (enzyme concentration, temperature, pH, substrate concentration and end-product concentration). In nature, the structure of substrate will limit the accessibility of enzyme to substrate, whereas, the hydrolysis conditions affected enzyme activity.

Basically, enzymatic hydrolysis consists three steps: adsorption of cellulase enzymes onto the surface of cellulose; the degradation of cellulose to fermentable sugars; and desorption of cellulase. Because of enzymatic hydrolysis is heterogeneous reaction and requires direct physical contact between enzymes and substrate, the enzymes must diffuse from bulk aqueous solution to the particle surfaces, diffuse through physical barrier (e.g. lignin), adsorb on substrate surface, and catalyze the hydrolysis (Chang and Holtzapple, 2000). Consequently, these reactions are complex and can be affected by physiochemical properties of substrate.

2.4.2.3.1   Crystallinity of cellulose

Cellulose crystallinity has often been inferred to affect hydrolysis rate (Mansfield et al., 1999, Converse, 1993). It seems logical that amorphous regions of the cellulose are hydrolysed first, and the crystalline regions are

degraded poorly (Walseth, 1952). It is widely accepted that decreasing the crystallinity increase the digestibility of lignocellulose (Fan et al., 1980). However, according to Puri (1984), crystallinity of cellulose is not a major resistant for enzymatic hydrolysis, because cellulase enzymes system contains enough enzyme groups to hydrolysis all the cellulose to glucose including the crystalline region cellulose.

Besides, both increased and decreased hydrolysis rate have been observed with increasing crystallinity of different lignocellulose substrates. Sinitsyn et al. (1991) reported there were negative correlations of cellulose crystallinity with efficiency of enzymatic hydrolysis, for pure cellulose substrates e.g. cotton linter, microcrystalline cellulose and α-cellulose. However, in case of baggase as substrate, the research group found that there was no strong relationship between crystallinity index and enzyme activity. Similar result was obtained by Kim and Holtzapple (2006) for pretreated corn stover. On the other hand, Yoshida et al. (2008) reported that decreased of crystallinity structure of *M. sinensis* (a perennial grass performing C4-type photosynthesis) will increase enzymes hydrolysis rate. From the results of different researches, may be more detail information on cellulose structure is needed, to find out which microstructures that influence the activity of cellulases.

2.4.2.3.2 Polymerization degree of cellulose

The degree of polymerization (DP) is defined as the number of glucosyl residues per cellulose chain (Mansfield et al., 1999). Therefore, a material with a lower DP has more cellulose chain ends. Endoglucanase (which act on cellulose chain of microfibrils and release chain ends) seems not to be

influenced by the DP. Exoglucanse or cellobiohydrolases which attack on chain end to produce cellobiose will exhibit a higher activity on substrates with low DP (Zhang and Lynd, 2004). The higher DP also shows higher crystallinity due to formation of more hydrogen bond inter and intra cellulose chains (Puri, 1984). On the other hand, Sinitsyn and co-workers studied the correlation between DP and hydrolysis rate of different substrates and found there was a very poor correlation between the DP and the initial rate of hydrolysis (Sinitsyn et al., 1990).

2.4.2.3.3   Presence of lignin and hemicelluloses

Cellulose, hemicelluloses and lignin are closely associated and covalent cross-linked have been suggested to occur between lignin and polysaccharides. The side groups arabinose, galctose and 4-O-methylglucuronic acid are most frequently perceived as connecting links to lignin (Fengel and Wengener, 1983). The presence of lignin and hemicelluloses makes the access of cellulose difficult, this reduce the efficiency of the hydrolysis (McMillan, 1994).

Grethlein (1985) has shown that the removal of hemicelluloses (by diluted acid pretreatment) results in an increasing of both the accessible pore volume and the specific surface area. He found that hydrolysis yield correlate with the pore volume accessible to a solute of 51 Å in size. Meanwhile, hemicellulose deacetylation has been shown to have minor role in enzymatic hydrolysis of pretreated poplar wood (Chang and Holzapple, 2000). Besides, hemicelluloses can be dissolved in diluted-alkaline and hot water. Thus, hemicelluloses can be isolated fromacid- treated and then dissolved in diluted-alkaline (Anis, 1995).

Lignin is responsible for integrity, structure rigidity and prevent of swelling of lignocellulose. Most of the literatures show that lignin content has impact on enzymatic hydrolysis. Lignin interfaces with hydrolysis by blocking access of cellulases to cellulose and by irreversible binding with hydrolytic enzymes (McMillan, 1994). Therefore, separation of lignin from the cellulose during pre-treatment is important to increase hydrolysis efficiency (Cowling and Kirk, 1976). Lignin content of lignocellulose is negatively correlated with enzymatic efficiency (Converse, 1993; Mansfield et al., 1999; Chang and Holtzapple, 2000). Delignification by sodium chlorite method, which is frequently used in initial step of holocellulose isolated from lignocellulose was carried out on *M. sinensis*, and try to relate delignification with hydrolysis rate (Yashida et al., 2008). They found that lignin is most significant resistant factor against the enzymatic hydrolysis of *M. sinensis*. Lu and co-workers also found that delignification on Douglas fir (softwood) to 8.2% of lignin content by stem-exploded with hot alkaline has increased yield of hydrolysis (Lu et al., 2002). On the other hand, partial lignin removal by alkaline sodium hydroxide treatment has resulted in decreased hydrolysis yields. The treatment partially dissolves the lignin in exploded substrates resulting in re-dispersal of lignin, occluding pores and smeared over cellulose surface, hence blocking pores and reducing digestibility (Wong et al., 1988; Donaldson et al., 1988). As conclusion, removal of lignin and hemicelluloses is important to increase porosity and accessible of cellulose.

2.4.2.3.4   Particles size / specific surface area of substrate

In natural, lignocellulose has both external (shape and siza of particles) and internal (capillary structure of the fiber) surface. In untreated lignocellulose