CHAPTER II
LITERATURE REVIEWS

This chapter contains information of lignocellulosic biomass, raw materials in this research, Technical Association of the Pulp and Paper Industry T203 test method, cellulosic ethanol properties and its use, the dilute acid hydrolysis and determination of total reducing sugars, yeast and biochemistry of cellulosic ethanol fermentation and determination of cellulosic ethanol, respectively.

2.1 Lignocellulosic biomass

The lignocellulosic biomass was described by Mohagheghi et al. (2004) that was a potential source of cheap sugars for producing fuels and chemicals, and a pretreatment stage was essential to make the cellulose accessible to the hydrolysis by dilute acid. The utilization of lignocellulosic biomass had been closely associated with a new technological concept, called as Biorefinery (Cazetta et al, 2007). Lim (2000) reported that the agricultural residue, softwood and hardwood compose the main components such as cellulose, hemicelluloses and lignin, as shown in Fig. 2.1. Their main components could determine with Technical Association of the Pulp and Paper Industry (TAPPI). The most use TAPPI T203 for determination of this lignocellulosic biomass. Moreover, they had great potential as cheap and renewable feedstock for cellulosic ethanol production (TAPPI, TAPPI, 1994-1995).

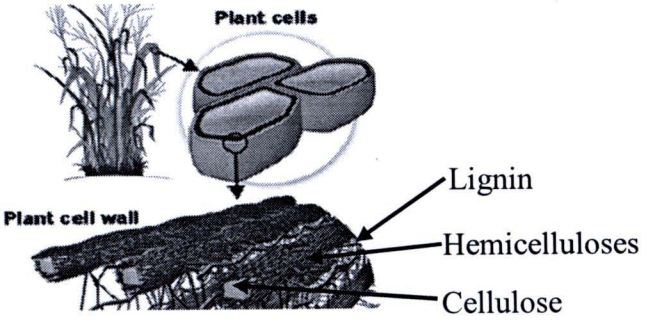


Figure 2.1 The main components of lignocellulosic biomass in plants (Lim, 2000)

2.1.1 Cellulose

The cellulose was reported by Nishiyama et al. (2002) that were an organic compound with the formula $(C_6H_{10}O_5)_n$. Minhee et al. (2011) described it consists of a linear chain of several hundred to over ten thousand β (1→4) linked D-glucose units and the chemical structure of cellulose was shown in Fig. 2.2. The cellulose was the structural component of the primary cell wall of green plants, many forms of algae and some species of bacteria secrete it to form biofilms (Yejun and Hongzhang, 2008). About 33 percentage of all plant matter was cellulose (Xu et al., 2003). Walker (1994) reported for the industrial processes, the cellulose was mainly obtained from wood pulp and cotton. It was mainly used to produce paperboard and paper. The converting cellulose from energy crops into biofuels such as cellulosic ethanol. It was under investigation as an alternative fuel source (Updegraff, 1969). Ayhan (2005) found that the cellulose fraction of various lignocelluloses was a uniform structure consisting of β-1,4 linked glucose units. However, the biodegradability of cellulose might vary between plants, depending on the strength of association of the cellulose with other plant compounds (Biely and Tenkanen, 1998).

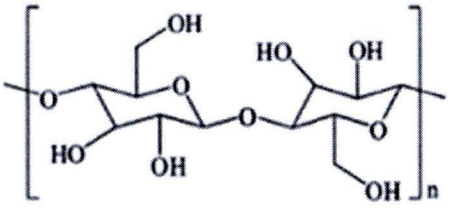


Figure 2.2 Chemical structure of cellulose (Minhee et al., 2011)

2.1.2 Hemicelluloses

The hemicelluloses were reported by Ebringerova et al. (2005) that was an organic compound which contains many different sugar monomers. Ming et al. (2007) described the sugar monomers in hemicelluloses could include xylose, mannose, galactose, rhamnose, arabinose and sugar acids. Hemicelluloses contain most of the D-pentose sugars and occasionally small amounts of L-sugars as well (Minhee et al, 2011). The xylose was always the sugar monomer present in the largest amount but mannuronic acid and galacturonic acid also tend to be present. Therefore,

the chemical structure was unlike the cellulose which shown in Fig. 2.3. The hemicelluloses consist of shorter chains 500-3,000 of sugar units as opposed to 7,000-15,000 of glucose molecules per polymer seen in the cellulose. In addition, hemicelluloses were a branched polymer (Nishiyama, 2002).

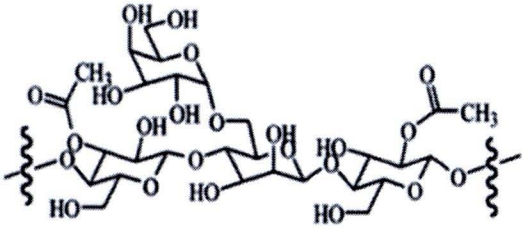


Figure 2.3 Chemical structures of hemicelluloses (Minhee et al., 2011)

2.1.3 Lignin

The lignin was showed by Boerjan et al. (2003) that was a complex phenol compound. The most commonly derived from wood and an integral part of the secondary cell walls of plants and some algae. The term was introduced in 1819 by de Candolle. It was derived from the Latin word lignum and meaning wood. It was one of the most abundant organic polymers on the Earth, exceeded only by cellulose, employing 30% of non fossil organic carbon and constituting from a quarter to a third of the dry mass of wood. As a biopolymer, the lignin was unusual because of its heterogeneity and lack of a defined primary structure. It was most commonly noted function was the support through strengthening of wood in trees (Walker, 1994). Minhee et al. (2011) shown the lignin had chemical structure which was a cross-linked raceme macromolecule with molecular masses in excess of 10,000 units which shown in Fig. 2.4.

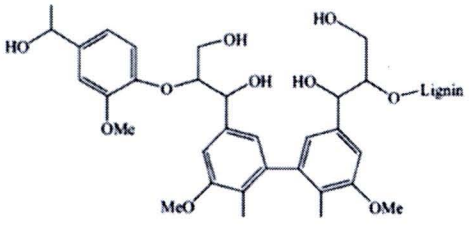


Figure 2.4 Chemical structure of lignin (Minhee et al., 2011)

2.2 Raw materials for cellulosic ethanol production

2.2.1 Durian

The durian (*Durio zibethinus* L.), King of fruits, was the fruit which was widely known and revered in Southeast Asia. It was distinctive for its large size, unique odour and formidable thorn-covered husk. It could grow as large as 30 cm of long, 15 cm of indiameter and it typically weighs 1-3 kg. Its shape ranges from oblong to round, the color of its husk green to brown and its flesh pale yellow to red, depending on the species was shown in Fig. 2.5. And moreover, the application of durian peels for daily life such as toothpaste, flowerpot, charcoal and paper. It most include of lignocellulosic biomass which could be raw materials to biofuel (O'Gara et al., 2004).

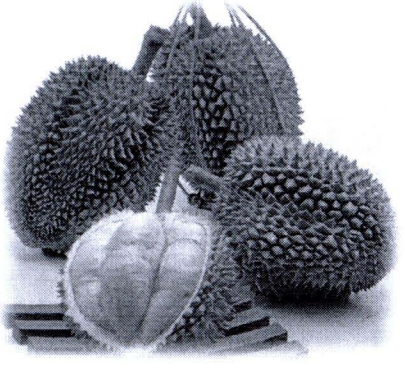


Figure 2.5 The photograph of meat and peel of durian (O'Gara et al., 2004)

2.2.2 Pineapple

The pineapple (*Ananas comosus*) was the common name for an edible tropical plant and its edible fruit which were coalesced berries. Pineapples were the only bromeliad fruit in widespread cultivation. It could be grown as an ornamental, especially from the leafy tops but would not fruit as shown in Fig. 2.6. Pineapple was eaten fresh or canned or juiced. It was popularly used in desserts and salads, as a complement to meat dishes and in fruit cocktail. The popularity of the pineapple was due to its sweet-sour taste containing 15% sugar and malic acid and citric fruit acids. It was also high in vitamin B1, B2, B6 and C. The protein digesting enzyme bromelin seems to help digestion at the end of a high protein meal. Pineapple peel was popular

to be component in common food of Northeast such as preserved fish. And moreover, it could be raw materials to biofuel due to it richly compose of lignocellulosic biomass (Jahn, 1990).

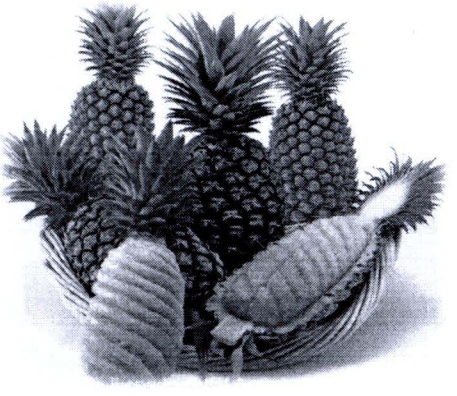


Figure 2.6 The photograph of meat and peel of pineapple (O'Gara et al., 2004)

2.3 TAPPI T203 test method

TAPPI (1994-1995) described that Technical Association of the Pulp and Paper Industry T203 test method or TAPPI T203 test method was the industrial method for preparation and determination of main components in lignocellulosic biomass such as lignin, hemicelluloses and cellulose. In each processes of TAPPI T203 test method could be removed the ester compound via mixture of Hexane and Methanol, lignin via Sodium chlorite, hemicelluloses via Sodium hydroxide. Finally, the cellulose was obtained. Therefore, this method was inclusively used for paper industrial.

2.4 Properties of the cellulosic ethanol and its use

Shakhashiri (2006) found that the cellulosic ethanol was the biofuel which could obtain from fermentation of lignocellulosic biomass. It had properties; chemical structure and its use were similarly ethanol as follow colorless liquid with a characteristic, agreeable odor. The chemical structure of ethanol or (CH_3CH_2OH) showed in Fig. 2.7. It was an alcohol, a group of chemical compounds whose molecules contain a hydroxyl group (-OH), bond to a carbon atom with a boiling

point of 78.5°C and melting point of -114.1°C. It was less than water with a density of 0.789 g ml^{-1} at 20°C.

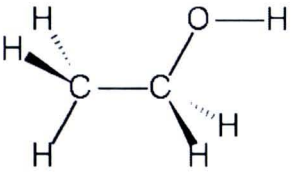


Figure 2.7 Chemical structure of ethanol (Morais et al., 1996)

The cellulosic ethanol was described by Lim (2000) that was one of the most exotic synthesis oxygen containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, antifreeze, a depressant, a fuel and especially because of its versatility as a chemical intermediate for other organic chemicals. Alcoholic beverages vary significantly in their ethanol content and in the foodstuffs from which they were produced such as beer, wine, whisky and gin. The alcoholic beverages could be mostly classified as fermented beverages. The beverages made by the action of yeast on sugary foodstuffs or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. Alcoholic beverages were sometime added to food in cooking, not only for their inherent flavor but also because the alcohol dissolves flavor compounds that water cannot. The cellulosic ethanol was an excellent transportation fuel which could be used as blend with gasoline, 10 and 22% blends were being used in the USA and Brazil, respectively. Cellulosic ethanol-blended with gasoline oxygenates it thereby reducing the formation of carbon monoxide and ozone (Laluce, 1991). The increase shortage of petroleum, urban air pollution and accumulation of carbon dioxide in the atmosphere, cellulosic ethanol was therefore expected to play a more significant role in the future. Cellulosic ethanol was frequently used to form blended gasoline fuel in concentrations between 10-85% (Wyman, 1994). In India, it had been made 5% ethanol-blending mandatory in petrol (The Gazette of India, 2002). In the next phase, supply of cellulosic ethanol-blended petrol would be extended to the whole country and efforts would be made to increase the percentage of cellulosic ethanol mixture in petrol to 10% (The Hindu, 2003).

Today almost cellulosic ethanol could be used as blend 85% (a blend of 85% cellulosic ethanol and 15% unleaded gasoline was E85 for use in Flexible Fuel Vehicles (FFVs)). It was very clean and even more environmentally friendly. E85 reduces harmful hydrocarbon and greenhouse gas emissions. E85 was the highest performance fuel which could be purchased at the retail level with its octane rating of at least 105 (American Coalition for Ethanol, 2007).

2.5 Dilute acid hydrolysis

The main challenges in producing cellulosic ethanol from renewable linocellulosic biomass had been found in hydrolysis stage. The hydrolysis of cellulose to glucose only occurs at economical viable yields when a catalyst was used. The main catalyst was diluting acid catalysts (Minhee et al., 2011). Water could then be added at low temperatures to dilute the acid solution, providing conditions to glucose. Wyman (1994) reported the lignocellulosic biomass had the potential to be a biomass feed stock for cellulosic ethanol production due to the high content of cellulose and hemicelluloses. Johanna et al. (2003) described the hemicelluloses could decompose at temperatures of approximately 160°C to form xylose and other sugars. The decomposition a temperature ranges of cellulose was 200-300°C. When the temperature more than 400°C, was usually required this generating problem with sugar degradation. At these temperatures, cellulose degrades into hydroxymethyl furfural and glucose converts into furfural, as shown in Fig. 2.8 (Badger, 2000).

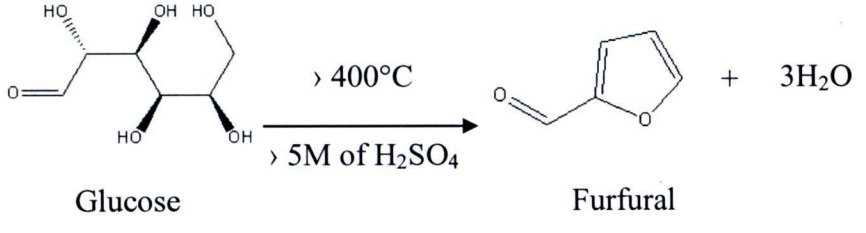
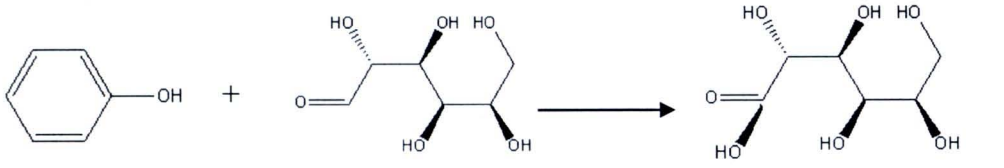


Figure 2.8 Chemical reactions of the reducing sugars (Badger, 2000)

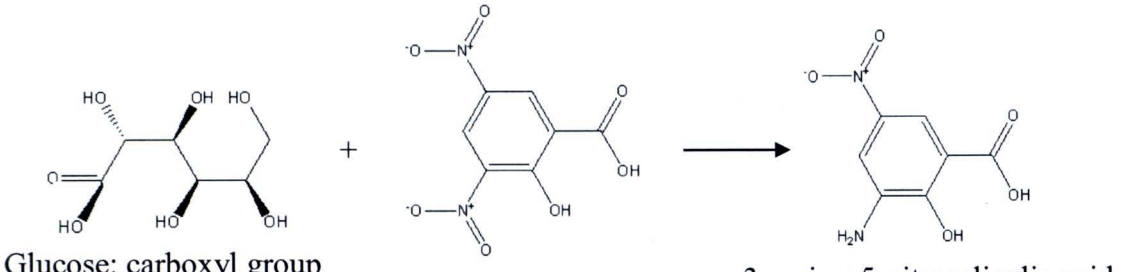
2.6 Dinitrosalicylic acid method

Miller (1959) reported the 3,5-Dinitrosalicylic acid (DNS or DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) was an aromatic compound that reacts with reducing sugars and other reducing molecules. The aldehyde group in reducing sugars was oxidized with phenol to carboxyl group, as shown in Fig. 2.9. Then carboxyl group was reduced with 3,5-Dinitrosalicylic acid to form 3-amino-5-nitrosalicylic acid which absorbs light strongly at 575 nm, as shown in Fig. 2.10. It was first introduced as a method to detect reducing substances in urine and had since been widely used for quantification of carbohydrates levels in blood. It was mainly used in assay of alpha-amylase. However, enzymatic methods were usually preferred to DNS due to their specificity.



Phenol Glucose: aldehyde group Glucose: carboxyl group

Figure 2.9 Chemical reactions of the glucose with phenol (Miller, 1959)



Glucose: carboxyl group 3,5-dinitrosalicylic acid 3-amino-5-nitrosalicylic acid

Figure 2.10 Chemical reactions of the glucose with DNS reagent (Miller, 1959)

2.7 Yeast and biochemistry

The cellulosic ethanol fermentation was found by Kosaric and Vardar (2001) that used microorganisms were selected to provide the best possible combination of characteristics for the process and equipment being used. The organism should have a high yield of product per unit substrate assimilated, high fermentation ability, substantial ethanol tolerance, and the ability to remain viable at high temperature, stability under adequate fermentation conditions and a tolerance to low pH values.

2.7.1 Yeast fermentation

The most commonly used microbe for cellulosic ethanol production was yeast. The fermentation of sugar to ethanol by yeast had an important among the difference processes that were used in fermentation (Kosaric and Vardar, 2001). The yeast strains of primary interest to industrial operations in fermentation of ethanol were *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Kluyueromyces sp* (Lim, 2000). Among the yeast strains, *S. cerevisiae*, which had a capability to produce cellulosic ethanol giving concentration as high as 18% in the fermentation broth, was in preferring. This yeast could growth both on simple sugars, such as glucose, and on the disaccharide sucrose. *S. cerevisiae* was also generally recognized as safe (GRAS) as a food additive for human consumption and was therefore ideal for producing alcoholic beverage and leavening bread. Yeast was very susceptible to cellulosic ethanol inhibition. Concentrations of 1-2% (w/v) were sufficient to retard microbial growth and at 10% (w/v) alcohol, the growth rate of the organism was nearly halted (Brown et al., 1981). Brown et al. (1981) reported that the immediate effects of this inhibition were more complex. Addition of ethanol to log phase yeast cultures resulted in rapid reduction of growth rate (possibly due to effects on protein synthesis), a decrease in cell variability and to a much less extent of ethanol lowers the rate of its own synthesis.

2.7.2 Biochemistry of fermentation processes

Thomas and Rose (1979) observed that the extent of ethanol tolerance for certain yeast strains was dependent upon the fatty acryl composition of their plasma membrane which would indicate that the fatty acryl composition favored or inhibited excretion of ethanol from the plasma. Yeast, under anaerobic conditions, metabolizes glucose to cellulosic ethanol primarily by way of the Embden-Meyerhof pathway. The overall net reaction involves the production of 2 moles each of cellulosic ethanol but the yield attained in practical fermentations however does not usually exceed 90-95% of theoretical (Lim, 2000). Yeasts are capable to utilize a variety of substrates. In general, they are able to grow and efficiently ferment cellulosic ethanol at pH values of 3.5-6.0 and temperature of 28-35°C. Though the initial rate of cellulosic ethanol production is higher at increased temperature (40°C) the overall productivity of the fermentation is decreased due to cellulosic ethanol product inhibition (Jones et al., 1981). Yeasts produce the ethanol through the Embden-Meyerhof (E-M) pathway, as shown in Fig. 2.11. The first three steps of the pathway prime (phosphorylate) and rearrange the hexose for cleavage into 2 trioses by the enzyme fructose 1,6-diphosphate aldolase, the key (cleavage) enzyme in the E-M pathway. Each triose molecule is oxidized and phosphorylated followed by two substrate level phosphorylations that yield 4 ATP during the drive to pyruvate to cellulosic ethanol and CO_2 which lactic acid bacteria reduce the pyruvate to lactic acid. The overall reaction is glucose $\longrightarrow$ cellulosic ethanol + CO_2 in yeast with a net gain of 2 ATP (Todar, 1998). The overall productivity of the fermentation is decrease due to enhanced cellulosic ethanol inhibition and this is a disadvantage of *S. cerevisiae* (Kosaric and Vardar, 2001).

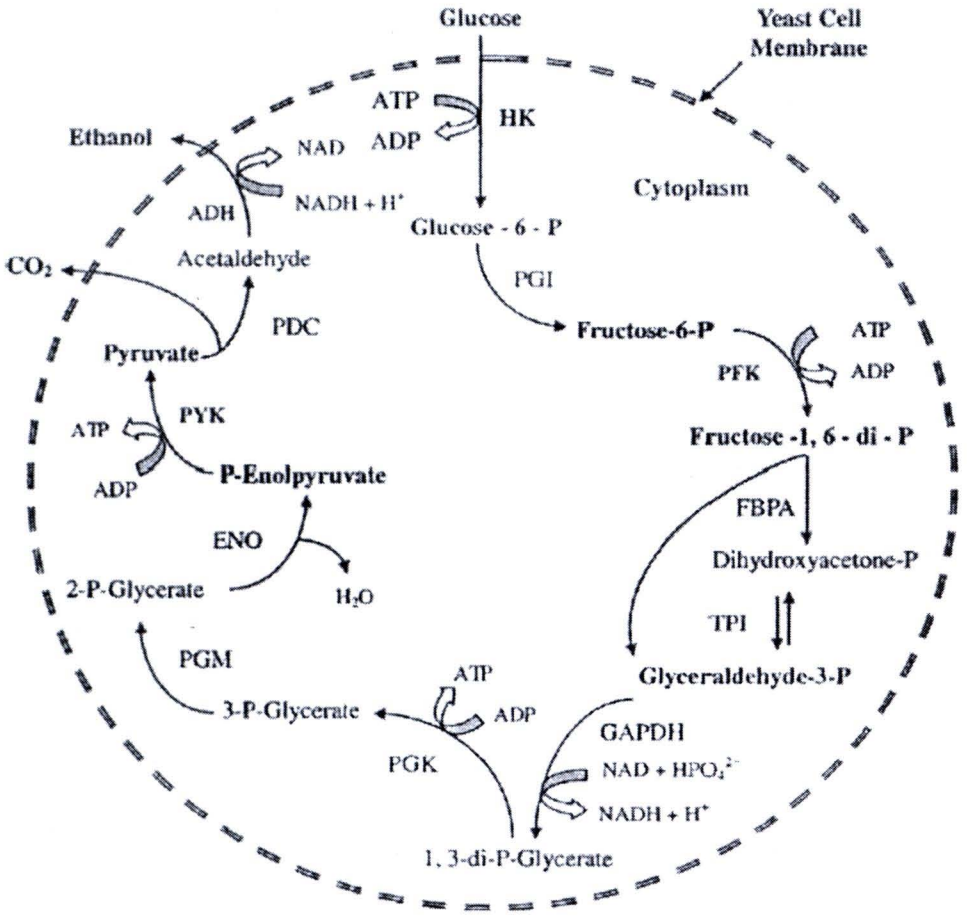


Figure 2.11 Embden-Meyerhof (E-M) pathway in anaerobic fermentation (Madugan et al., 2000)

2.8 The factors and yeast nutrition

The cellulosic ethanol could be obtained with the help of a fermentation method from raw materials containing polysaccharides factor and yeast nutrition of cellulosic ethanol production (Marek et al., 2007).

2.8.1 Factors affecting on cellulosic ethanol production

2.8.1.1 Substrate

Fermentation processes from any material that the sugar could derive cellulosic ethanol. The varied raw materials used in the manufacture of cellulosic ethanol via fermentation were conveniently classified into three main types

of raw materials: sugars, starches and cellulose materials. Sugars (from sugarcane, sugar beets, molasses and fruits) could be converted into ethanol directly. Starches (from corn, cassava, potatoes and root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt and molds. Cellulose (from wood, agricultural residues, waste sulfite liquor from pulp and paper mills) must likewise be converted into sugars, generally by the action of mineral acids. Once simple sugars were formed, enzymes from microorganisms could readily ferment them to ethanol (Lin and Tanaka, 2006).

2.8.1.2 pH

In cellulosic ethanol fermentation, the pH of medium was in the range of 4.0-5.5 which was a possible way to control bacterial contamination during the fermentation (Narendranath and Power, 2005). The pH of medium was an important factor for ethanol yield. Lui and Shen (2008) reported that pH 5.0 was appropriate for ethanol production from stalk juice of sweet sorghum by immobilized *S. cerevisiae*.

2.8.1.3 Oxygen

Glycolysis was the major pathway for sugar utilization by yeast. Under excess oxygen condition, pyruvate is transformed to carbon dioxide and water resulting in high cell concentration. On the other hand, under anaerobic condition, pyruvate was converted into ethanol and this process was called fermentation. Grosz and Stephanopoulos (1990) studied the control of glycerol production under various oxygen levels. They found that the presence of low oxygen levels clearly reduced glycerol production compared to that under anaerobic culture.

2.8.2 Yeast nutrition

The main nutrients which were important for cellulosic ethanol fermentation, as follow:

2.8.2.1 Nitrogen

The composition of yeast cells contains nitrogen about 10% of their dry weight (Walker, 1994). Therefore, nitrogen was the important constituents

for yeast growth. Ammonium nitrogen in the form of NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$ were commonly used as a nitrogen source for growth (Berry et al., 1987). The requirement of nitrogen for protein synthesis might be met by ammonium ion, although amino acids were a preferred source. A wide range of substances (amino acid, peptides, urea, purine, pyrimidines and amines) were able to serve as the nitrogen sources. Amino acids were taken up in a sequential manner during fermentation (Jones et al., 1964).

2.8.2.2 Phosphorus

Phosphorus was assimilated only in the form of the orthophosphate ion ($H_2PO_4^-$). It was a component of sugar phosphate, nucleic acid, nucleoside di or triphosphate and phospholipids. Therefore, phosphate was essential for all yeasts. In addition, condensed inorganic phosphates were a common source of phosphorus in yeast growth media (Walker, 1994).

2.8.2.3 Sulphur

Sulphur was incorporated into S-containing amino acid in protein. The sulphur content in yeast cells represents around 0.3% of cell dry weight (Walker, 1994). Methionine was the most effectively used amino acid in yeast nutrition. It allows greater and more rapid growth than SO_4^{2-} (Hough, 1971).

2.8.2.4 Magnesium

Magnesium was the cofactor or effectors for many enzymes in yeast metabolism. It involves in many cellular processes such as activation of glycolysis enzymes, stimulation of essential fatty acid synthesis, activation of membrane ATPase, regulation of cellular of ionic level and along with K^+ involved with phosphate uptake. The main sources of magnesium were from $MgCl_2$ and $MgSO_4$ (Berry et al., 1987).

2.9 Fermentation processes

In industrial operations, ethanol production could be produced by batch, fed-batch and continuous processes (Caylak and Vardar, 1998).

2.9.1 Batch process

In batch fermentation, the fermentation medium and yeast culture were added into the bioreactor together with nutrients without the removal of culture medium during the fermentation (Lin and Tanaka, 2006). The products were harvested at the end of the fermentation. Presently, most of the ethanol produced was performed by the batch fermentation because the investment costs were low and the system does not require much control and could be accomplished with unskilled labour. Complete sterilization and management of feed stocks were easier than in other processes. Another advantage of batch operation was the great flexibility that could be achieved by using a bioreactor for various product specifications (Caylak and Vardar, 1998).

2.9.2 Fed-batch process

In fed batch fermentation, the fresh fermentation medium was added to a fermented sequentially without removal of the fermentation medium and the product was harvested at the end of the fermentation. The process was widely used for the production of microbial biomass, ethanol, organic acid, antibiotics, vitamins, enzymes and other compounds (Stanbury et al., 1995).

2.9.3 Continuous process

In continuous processes, feed which contain substrate, the culture medium and other required nutrients were pumped continuously into an agitated vessel where the microorganisms were active. The product which was taken from the top of the bioreactor contains ethanol, cells and residual sugar (Maiorella et al., 1984). Unfortunately, continuous fermentation was more susceptible to long-term bacteriological problem. However, this process for ethanol fermentation was used in Thailand by Thai Agro Energy, Suphan buri, Thailand (Bayrock and Ingledew, 2001).

2.10 Gas Chromatograph equipped with a Flame Ionization Detector

Gas Chromatography (GC) was a common type of chromatography used in analytic chemistry for separating and analyzing compounds that could be vaporized without decomposition. In some situations, it might help in identifying a compound. The mobile phase was a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase was a microscopic layer of liquid or polymer on an inert solid support, inside a column. Flame Ionization Detector (FID) detects the analysts by measuring an electrical current generated by electrons from burning carbon particles in the sample. It was a non-selective detector used in conjunction with gas chromatography. There was a potential for many non-target compounds present in samples to interfere with this analysis (Lee et al., 2006). The literatures of the ethanol production in 2003 to 2011 contain raw material, fermentation processes, microorganism for fermentation and determination of ethanol, as shown in Table 2.1.

Table 2.1 Literatures on production and determination of ethanol

Material	Fermentation	Microorganism	Method	References
Spruce	SSF	*S. cerevisiae*	HPLC	Johanna et al. (2003)
OCC, Paper sludge	SSF	*K. marxianus* Y01070	HPLC	Zs. Kardar et al. (2004)
Finger millet flour	VGH	*S. cerevisiae*	GC-FID	Reddy et al. (2006)
Fresh spruce	SSF	*S. cerevisiae* TMB3000	HPLC	Malek A. et al. (2006)
Corncob	Fed-batch fermentation	*S. cerevisiae*	HPLC	Ming et al. (2007)
Corn stover	SSF	*S. cerevisiae*	HPLC	Karin et al. (2007)
Paper sludge	SSF and SHF	*Pichia stipitis*	HPLC	Marques S. et al (2008)
Wheat straw	SSF	*S. cerevisiae*	HPLC	Marie L. et al (2008)
Fresh corn	SSF	*S. cerevisiae*	HPLC	Yejun H. et al. (2008)
Wheat straw	SSF and Fed-batch fermentation	*K. marxianus* CECT 10875	GC-FID	Thomas E. et al (2009)
Cassava stem	Batch fermentation	*S. cerevisiae* CHY 1011	HPLC	Minhee et al. (2011)
Groundnut shell	Batch fermentation	*Pichia stipitis* NCIM 3498	GC-FID	Chandra et al. (2011)