CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Ethanol

Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. Its current applications include potable, chemical, and fuel ethanol. Ethanol (also known as ethyl alcohol) is the most common of alcohols. It is the form of alcohol that is in alcoholic beverages and is easily produced from corn, sugar, or fruit through fermentation of carbohydrates. Its chemical structure is CH_3CH_2OH. It is less toxic than methanol. Ethanol is a colorless liquid with a melting point of -144°c and a boiling point of 78 °c. It is less dense than water with a density of 0.789 g/ml and soluble at all concentrations in water. Ethanol is frequently used to form blended gasoline fuels in concentration between 10-85%. More recently, it has been investigated as a fuel for direct ethanol fuel cells (DEFC) and biofuel cells. Ethanol was deemed the "fuel of the future" by Henry Ford and has continued to be the most popular alcoholic fuel for several reasons (Shelley, 2006):

(i) It is produced from renewable agricultural products (corn, sugar, molasses, etc.) rather than nonrenewable petroleum products.

(ii) It is less toxic than the other alcohol fuels.

(iii) The incomplete oxidation by-products of ethanol oxidation (acetic acid (vinegar) and acetaldehyde) are less toxic than the incomplete oxidation by-products of other alcohol oxidation.

Mixing ethanol with gasoline has several advantages. The higher octane number of ethanol (96–113) increases the octane number of the mixture. Ethanol also provides oxygen for the fuel, which will lead to the reduced emission of CO and non-combusted hydrocarbons (Galbe *et al.*, 2002).

2.1.1 Ethanol Production

The production of ethanol can be categorized base on its production route to be synthetic ethanol and bioethanol.

(i) Synthetic ethanol:

Ethanol is manufactured by the direct hydration of ethylene (the petroleum product). The reaction is reversible, and the formation of the ethanol is exothermic (Clark, 2002).

$$CH_2{=}CH_{2\,(g)} + H_2O_{\,(g)} \rightleftharpoons CH_3CH_2OH_{\,(g)} \qquad \Delta H = -46\ kJmol^{-1}$$

(ii) Bioethanol:

Ethanol is produced via biological processes. Production of bioethanol involves the conversion of a feedstock crop into fermentable sugars through enzyme amylases. Yeast is then added to ferment the sugars into ethanol and carbon dioxide. There are many feedstock crops which are used for bioethanol production such as corn, sugarcane bagasse, potato, cassava, cellulosic biomass (Kevin *et al.*, 2006).

2.2 Fermentation

Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as molds, yeasts, or bacteria. Enzymes act by hydrolysis, a process of breaking down or predigesting complex organic molecules to form smaller (and in the case of foods, more easily digestible) compounds and nutrients. Examples of important enzymes are (William *et al.*, 2007):

(i) The enzyme protease breaks down huge protein molecules first into polypeptides and peptides, then into numerous amino acids, which are readily assimilated by the body.

(ii) The enzyme lipase hydrolyzes complex fat molecules into simpler free fatty acids.

(iii) The enzyme amylase works on carbohydrates, reducing starches and complex sugars to simple sugars.

2.2.1 Ethanol Fermentation

Ethanol fermentation also referred to alcoholic fermentation, is a biological process in which sugars such as glucose, fructose, and sucrose are converted into ethanol, energy and carbon dioxide by the enzyme produced from yeast cell. The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), though which one molecule of glucose is metabolized, and two molecules of pyruvate are produced as illustrated in the equation (Madigan *et al.*, 2000). The free energy released in this process is used to form the high-energy compounds ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Glycolysis is summarized by the chemical equation:

(glucose) (pyruvate)

$$C_6H_{12}O_6 + 2ADP + 2P_i + 2NAD^+ \rightarrow 2CH_3COCOO^- + 2ATP + 2NADH + 2H_2O + 2H^+$$

The pyruvate is further reduced to ethanol with the release of CO_2. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth, which means yeast must be produced as a co-product. Without the continuous consumption of ATPs by the growth of yeast cells, the glycolytic metabolism of glucose will be interrupted immediately, because of the intracellular accumulation of ATP, which inhibits phosphofructokinase (PFK), one of the most important regulation enzymes in the glycolysis (Bai *et al.*, 2008). Figure 2.1 shows the metabolic pathway of ethanol fermentation by *Saccharomyces serevisiae*.

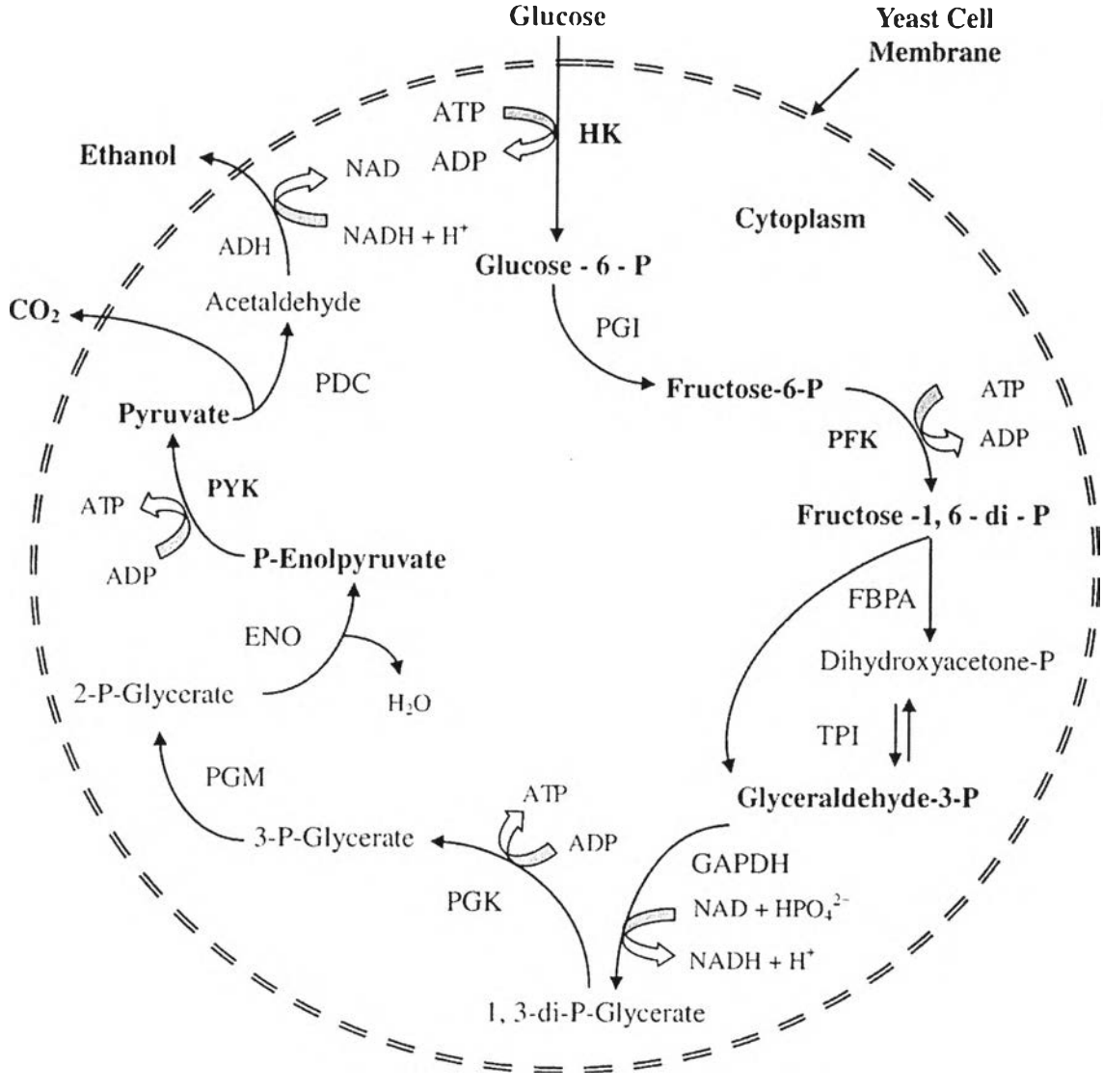
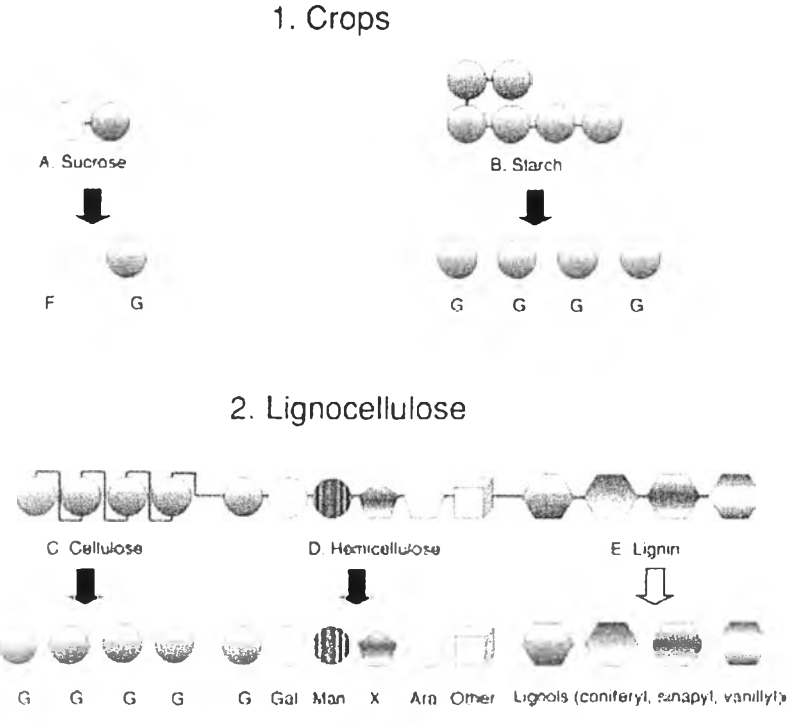


Figure 2.1 Metabolic pathway of ethanol fermentation in *Saccharomyces serevisiae* (Madigan *et al.*, 2000).

Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

A variety of sources can provide sugars for bioethanol fermentation (ethanol fermentation from biomass), including crops and lignocelluloses (as shown in Figure 2.2). Crops such as sugar cane and sugar beet contain sucrose, which can be converted into its monomeric components and other crops such as corn and cereals contain starch, which can be also converted into glucose. Moreover the utilization of a cheaper substrate such as lignocelluloses could make bioethanol (Zaldivar *et al.*, 2001).



ure 2.2 Sources of sugars for ethanol production. 1 Crops; 2 lignocellulose - ›ws represent hydrolysis (only monomers generated from hydrolysis, represented lark arrows, can be fermented). G Glucose, Gal galactose, F fructose, Man ıose, X xylose, Ara arabinose, Other L-rhamnose, L-fucose, uronic acids.

During ethanol fermentation, yeast cells are progressively exposed to a very stressful environment due to the strong decrease of external pH, the rising accumulation of ethanol in the medium and nutrient depletion, which leads to a sudden fall of cellular viability of yeast cell before ethanol fermentation is completed (Salmon *et al.*, 1996). So there are several improvements in order to enhance efficiency of ethanol fermentation such as improvement of microorganisms strain, using continuous fermentation instead of a batch type.

2.2.2 Continuous Ethanol Fermentation

For continuous ethanol fermentation, fresh medium is continuously added into the reactor and at the same time an equal amount of the fermented medium is continuously removed. (Clark, 2010). Model of continuous fermentation system are showed in Figure 2.3.

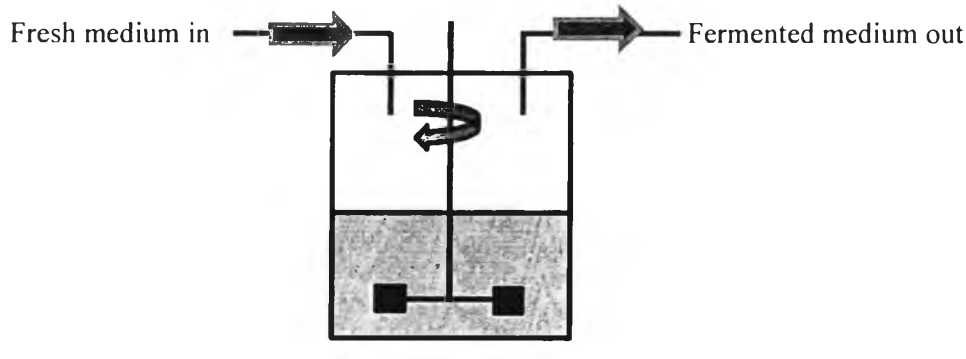


Figure 2.3 Model of continuous fermentation system.

Continuous ethanol fermentation provides important economic advantages and significantly improves production rate. It provides advantages over other systems (Bayrock *et al.*, 2001).

1. Long- term continuous productivity
2. High volumetric productivity
3. High conversion rate

Ghorbani *et al.* (2011) investigated the use of calcium-alginate immobilized yeast to produce ethanol continuously using cane molasses as a carbon source. Figure 2.4 showed effect of sugar concentration on ethanol production. The ethanol production was done by the concentration of the cane molasses (50, 100 and 150 g/l), dilution rates (0.064, 0.096, 0.144 and 0.192 h^{-1}) and hydraulic retention time (5.21, 6.94, 10.42 and 15.63 h) of the media. The pH of the feed medium was set at 4.5 and the fermentation was carried out at an ambient temperature. They found that fermentation with high concentration of total sugar and high retention time (HRT) produced high levels of ethanol.

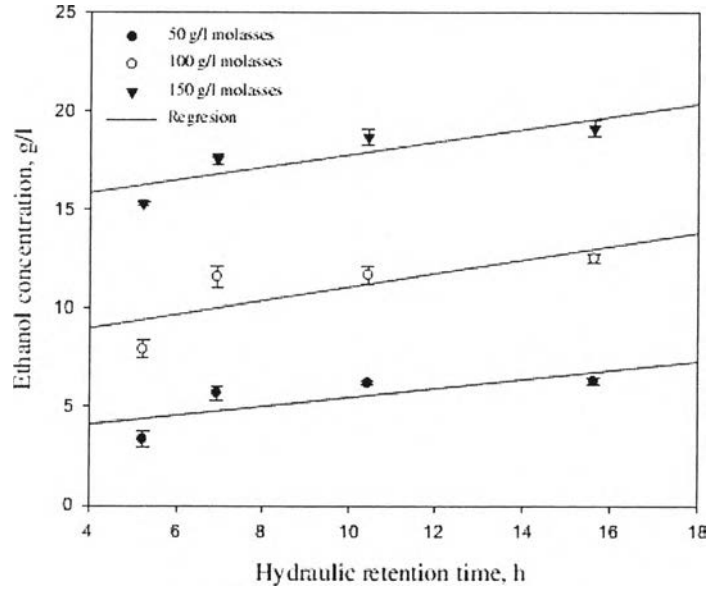


Figure 2.4 Effect of sugar concentration on ethanol production in a continuous immobilized cells reactor with different HRT.

Another technique for enhancing efficiency of ethanol fermentation is using immobilization cells method. It is the most widely used. Using immobilization cells method can increase cell mass concentration in bioreactor and improve the efficiency of substrate utilization and productivities of fermentation processes with minimum of the production costs (Vesna *et al.*, 2012).

2.3 Cell Immobilization System

Cell immobilization has been defined as "the physical confinement or localization of intact cells to a certain defined region of space with preservation of some desired catalytic activity" (Karel *et al.*, 1985). Immobilization normally involves attachment of the biocatalyst to, or location within, an insoluble carrier. Many biotechnological processes could benefit from immobilization of biocatalysts. Immobilization offers many potential advantages over free cell systems as following (Nedovic *et al.*, 2001).

(i) Higher cell densities and cell loads.
(ii) Increased volumetric productivity.
(iii) Simple separation of cell from product after reaction.
(iv) Reuse of the same biocatalysts for prolonged periods of time due to constant cell regeneration.
(v) Improved substrate utilization.
(vi) Reduced risk for microorganism contamination.
(vii) Simplified process design.
(viii) Improved tolerance to end products and protection of cells.
(ix) Protection of cell from shear force.

Therefore, using immobilized cell systems are widely used for different mentation processes such as beer (Nedovic *et al.*, 1997, Pilkington *et al.*, 1998), ne (Divies *et al.*, 1994; Yokotsuka *et al.*, 1997) and cider (Durieux *et al.*, 1998; lovic *et al.*, 2000) fermentations.

Cell immobilization techniques can be divided into four major groups based on physical mechanisms of immobilization (Pilkington *et al.*, 1998): adsorption to a pre-formed carrier, physical entrapment within a porous matrix, self aggregation in floes and containment of cells behind a barrier.

2.3.1 Adsorption to a Pre-formed Carrier

The earliest type of cell immobilization is based on cell adsorption onto external surfaces of solid carriers. Cells can be attached by Van der Waals forces, electrostatic interactions between oppositely charged surfaces, covalent bonding and physical entrapment in the pores of carrier (Nedovic *et al.*, 2001). For the adsorption of yeast cells various materials proved suitable, which can be divided in two general types; Figure 2.5A, materials with the yeast cells restricted to the external surfaces only, and Figure 2.5B, materials with pores large enough to allow cell adsorption inside the material.

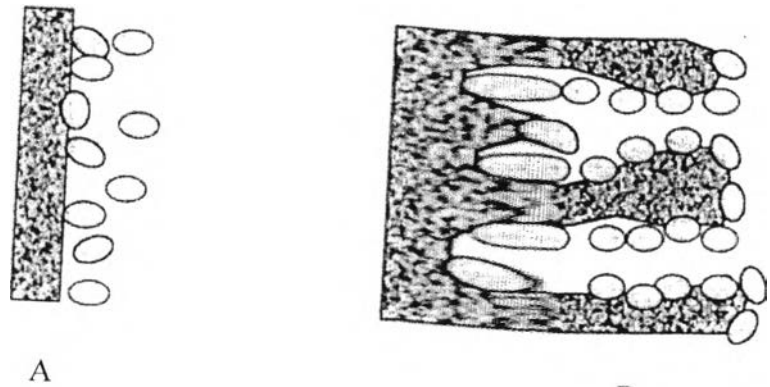


Figure 2.5 Adsorption to a per-formed carrier (Nedovic *et al.*, 2001).

2.3.2 Cell Entrapment

Cell immobilization by entrapment (as shown in Figure 2.6) is based on porosity of matrix that at the same time retains cells within the carrier and provides metabolite diffusion. Alginate, *kappa*-carrageenan and pectate gels in shape of spheres were mostly used as matrix materials for yeast immobilization (White and Portno, 1978; Pardonova *et al.*,1982; Hsu and Bernstein, 1985; Onaka *et al.*, 1985; Curin *et al.*, 1987; Nedovic *et al.*, 1993). Main advantage of the cell entrapment method is attainment of extremely high cell loading providing high fermentation rates. However, in some cases cell proliferation and activity can be limited by low mass transfer rates within the matrices.

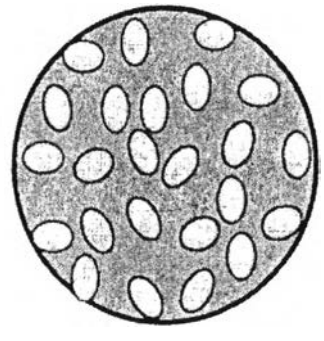


Figure 2.6 Cell entrapment within the carrier (Nedovic et al., 2001).

2.3.3 Self-aggregation

Self-aggregation of cells (as shown in Figure 2.7) can be natural or artificially induced by crosslinking agents. This technique is based on the use of highly concentrated suspensions of flocculent yeast strains. Although this is the simplest and least expensive immobilization method, it is the most sensitive to the changes in the operating conditions. In addition, there is a high risk of cell wash-out from the system (Nedovic *et al.*, 2001).

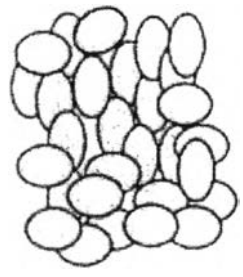


Figure 2.7 Self-aggregation of cells (Nedovic *et al.*, 2001).

2.3.4 Containment of Cells behind a Barrier

This cell immobilization technique cells are confined to a space bounded by a semi-permeable barrier or immobilized within a membrane. There are very little data on yeast immobilization for beer fermentation by this method (Nedovic et al., 2001). Figure 2.8 shows containment of cells behind carrier.

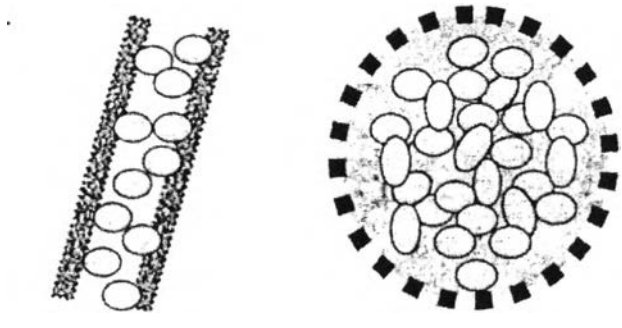


Figure 2.8 Containment of cells behind carrier (Nedovic *et al.*, 2001).

Selection of carriers and method of immobilization is made by considering the various characteristics and required features of the biocatalyst application against the properties, limitations and characteristics of the combined immobilization support. There are many researches that use the yeast cells immobilization technique in order to increase their productivity efficiency.

Johansen *et al.* (1986) investigated a method for immobilizing yeast cells in alginate particles by internal gelation. The internal gelling method for immobilizing cells in calcium alginate gels appears to be a promising alternative to the traditional external gelling method. With this method it is possible to manufacture gel particles of almost any shape. In addition the possession of a relatively high fermentation rate at small surface areas and a gel strength that does not decrease during fermentation.

Fujii *et al.* (1999) studied influence of surface characteristics of cellulose carriers on ethanol production by immobilized yeast cells. A positive charge of the carrier is proper for yeast cells immobilization because it involves with the interaction between the cells and the carrier. Modification of the cellulose carrier with diethylaminoethyl (DEAE) was also studied. It was a suitable function group for immobilization and ethanol production. As a result of good growing yeast cells immobilized on porous cellulose carriers, ethanol production was carried out. SEM image of immobilized cells on BC after the immobilization culture was shown in Figure 2.9.

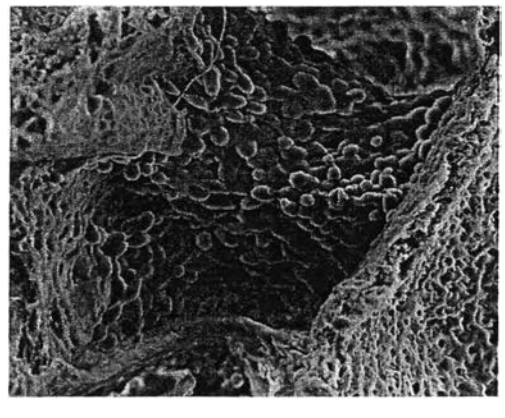
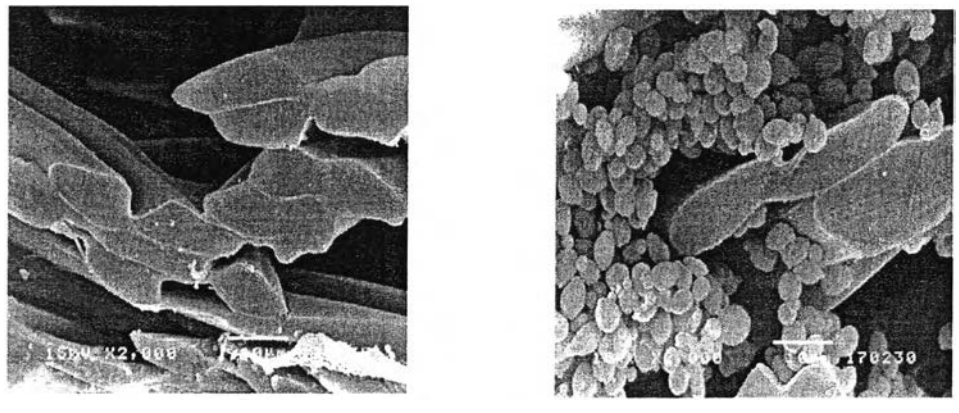


Figure 2.9 Scanning electron micrograph of immobilized cells on BC after the immobilization culture (Fujii *et al.*, 1999).

Rattanapan *et al.* (2011) studied using a thin-shell silk cocoon (TSC), a residual from the silk industry, as a support material for the immobilization of *Saccharomyces cerevisiae* M30 in ethanol fermentation. There are many advantages for immobilized yeast cells in TSC such as reusability, altered mechanical strength, cell regeneration and high immobilized yield, were achieved and resulted in stable operation with a high ethanol production and high biomass density. Figure 2.10A and 2.10B showed a cross-section of a thin-shell silk cocoon (TSC) at the initial time (0 h) and after the five-cycle repeated batch fermentation (240 h), respectively.



A B

Figure 2.10 A cross-section of a thin-shell silk cocoon (TSC) at the initial time (0 h), (A) and after the five-cycle repeated batch fermentation (240 h), (B) (Anuchit *et al.*, 2011).

Vesna *et al.* (2012) investigated the potential of sugar beet pulp (SBP) and dried sugar beet pulp (DSBP) as economically cheap and renewable supports for immobilization of *Saccharomyces cerevisiae* cells. The DSBP-immobilized biocatalyst was used for repeated ethanol fermentation because DSBP is more stable material. This study demonstrates that the efficient bioethanol production from sugar beet thick juice in repeated batch mode using *Saccharomyces cerevisiae* immobilized on sugar beet pulp is possible even without any nutrient supplementation. Yeast cells immobilization was confirmed by the scanning electron microscopy (SEM), as shown in Figure 2.11.

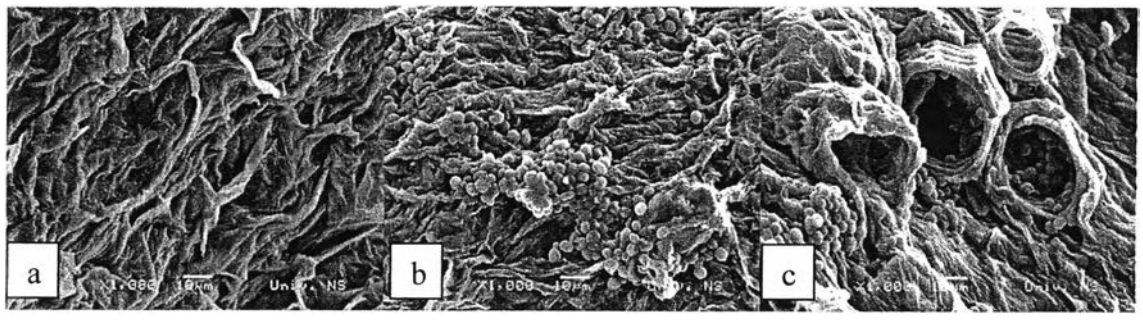


Figure 2.11 Scanning electron microphotograph (1000x) of the (a) hydrated DSBP; *Saccharomyces cerevisiae* immobilized (b) on the surface and (c) inside the cavities of hydrated DSBP (Vesna *et al.*, 2012).

2.4 Silk Fibroin (SF)

Silk, often called "The Queen of Fibers," is made from the cocoons of silk producing moths. The silk from the cocoon of silkworm *Bombyx Mori* is the most studied. It is a continuous protein fiber that is synthesized in the silk glands of the silkworm where its secreted and stored in the lumen, thereafter its transformed into fibers by the stretching of the liquid silk through the head movement of the silkworm (Ayutsede, 2005). Figure 2.12 shows physical structure of raw silk fiber.

Normally, native silk fiber consists of two types of self-assembled proteins: fibroin and sericin. Table 2.1 shows composition of silk produced by *Bombyx mori* silkworm Two strands of fibroin are bonded together to form a core structure located in the center of the silk fiber to provide the strength. The core fibroins are encased in a coat of sericin, a family of hydrophilic proteins, which acts as a natural glue for maintaining the shape of cocoon (Wu *et al.*, 2007) but concealing the unique luster of silk. These two proteins contain the same 18 amino acids such as glycine, alanine and serine in different amounts, as shown in Table 2.2 (Cao *et al.*, 2009).

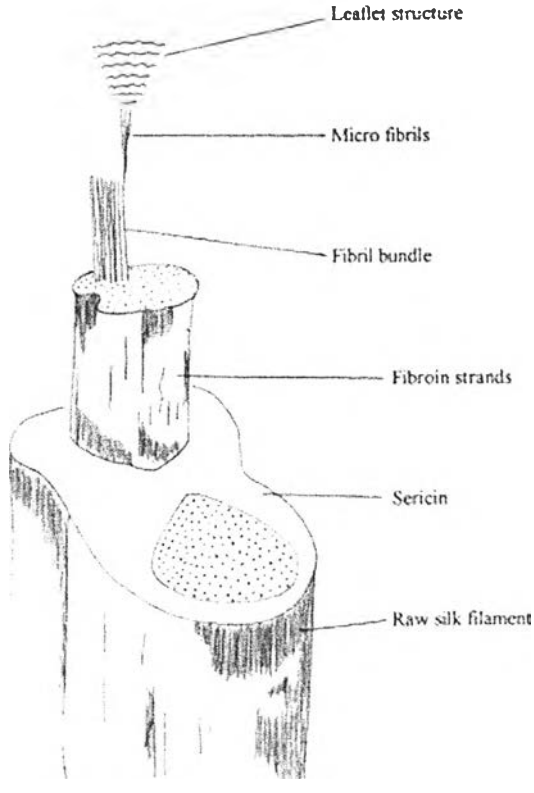


Figure 2.12 Physical structure of raw silk fiber (Sandoz, 1990).

Table 2.1 Composition of silk produced by *Bombyx mori* silkworm (Gulrajani, 1988)

Component	**Percent (%)**
Fibroin	70 – 80
Sericin	20 – 30
Wax matter	0.4 – 0.8
Carbohydrates	1.2 – 1.6
Inorganic matter	0.7
Pigment	0.2
Total	100

Table 2.2 Amino acid composition of *Bombyx mori* fibroin (Shimura *et al.*, 1982)

Amino acid	Composition, mol%
Glycine	42.9
Alanine	30.0
Serine	12.2
Tyrosine	4.8
Valine	2.5
Aspartic acid	1.9
Glutamic acid	1.4
Threonine	0.92
Phenylalanine	0.67
Methionine	0.37
Isoleucine	0.64
Leucine	0.55
Proline	0.45
Arginine	0.51
Histidine	0.19
Lysine	0.38

Sericin is insoluble in cold water but it is easily hydrolyzed, where by the long protein molecules break down to smaller fractions, which are easily dispersed, or solubilized in hot water (Gulrajani, 1988). The sericin removal process is called "silk degumming". After degumming, the leftover is silk fibroin made up of two strands. Silk fibroin can be used for many purposes including textile, medical and industrial applications (Mondal *et al.*, 2007). Silk must be degummed for biomedical and biological applications in order to remove the immunogenic sericin coating (Kearns *et al.*, 2008).

2.4.1 Structure of SF

2.4.1.1 Primary Structure

SF is an insoluble protein containing up to 90 % of the amino acids, like glycine (Gly), alanine (Ala), and serine (Ser). The primary structure of *Bombyx mori* silk fibroin has been determined to be composed dominantly of a six-amino acid-residue motif, i.e.,-Gly-Ala-Gly-Ala-Gly-Ser-, as shown in Figure 2.13 (Mita *et al.*, 1988).

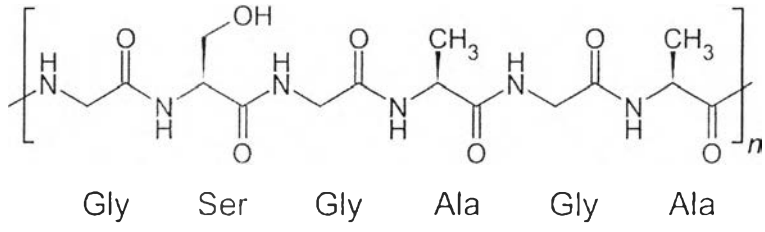
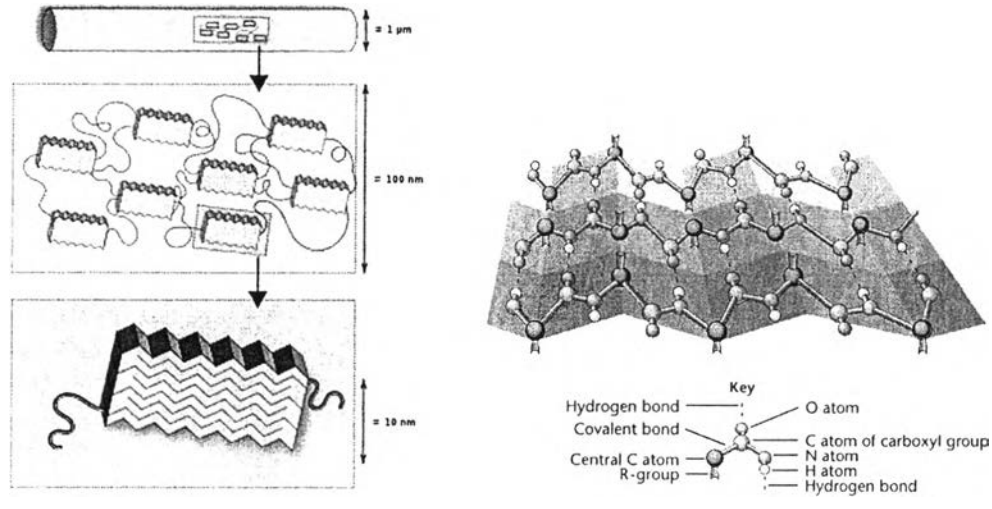


Figure 2.13 Primary structure of SF.

2.4.1.2 Secondary Structure

Silk fibroin has main conformations in the solid state, which are random coil conformation (silk I) and β-sheet structure (silk II) (Yang *et al.*, 2009). The latter conformation (silk II) is the solid fibroin that can be found in the spun silk thread while silk I is a meta-stable form stored in the silk gland of the silkworms. The silk I structure is the water-soluble state. The silk I structure is observed in vitro in aqueous conditions. The β-sheet structures are asymmetrical with one side occupied with hydrogen side chains from glycine and the other occupied with the methyl side chains from the alanines that populate the hydrophobic domains. The β-sheets are arranged so that the methyl groups and hydrogen groups of opposing sheets interact to form the inter-sheet stacking in the crystals. Strong hydrogen bonds and van der Waals forces generate a structure that is thermodynamically stable. The inter- and intra-chain hydrogen bonds form between amino acids perpendicular to the axis of the chains and the fiber. The silk II structure is insoluble in water and several solvents (Vepari *et al.*, 2007). Figure 2.14a and

2.14b show structure of SF composed of amorphous and crystalline region and β-sheet conformation of the crystalline region of SF, respectively.

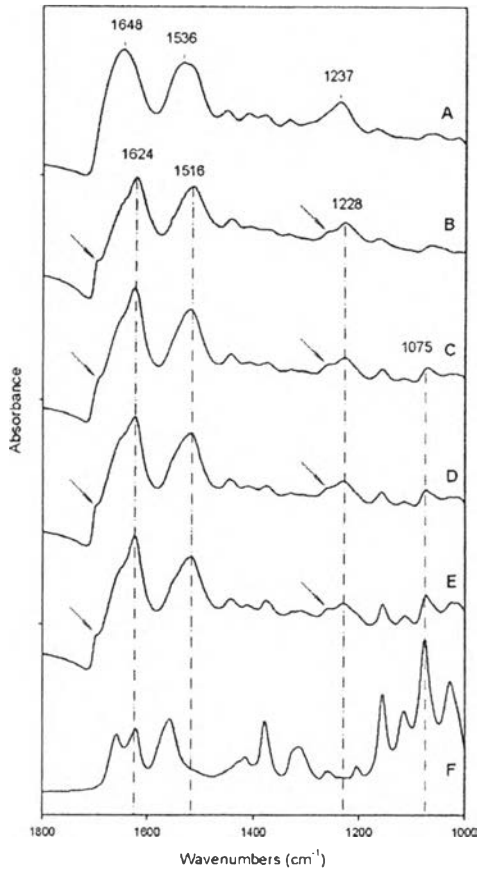


(a) (b)

Figure 2.14 (a) Structure of SF composed of amorphous and crystalline region and (b) β-sheet conformation of the crystalline region of SF.

The β-sheet structure of silk fibroin can be induced by several treatments such as thermal treatment, chemical cross-linking agents treatment (Mingzhong *et al.*, 2003), shear and elongation forcing (Fang *et al.*, 2006), methanol treatment, blending with other polymers (chitosan (Chen *et al.*, 1997), sodium alginate, poly(ethylene glycol) (Cilurzo *et al.*, 2011)).

Wongpanit *et al.* (2007) used methanol to induce the β-sheet formation of silk fibroin sponge to make the water-stable materials. To improve dimensional stability of silk fibroin sponge, chitin whiskers were incorporated as nanofiller. The silk fibroin sponges were immersed in an aqueous methanol solution at a concentration of 90% (v/v) for 10 min. After the silk fibroin sponge was treated with methanol, the peaks of amide I and amide II were shifted to 1624 and 1516 cm^{-1}, respectively and a new absorption shoulder was observed, which are the characteristic absorptions of β-sheet form of silk fibroin (as shown in Figure2.15).



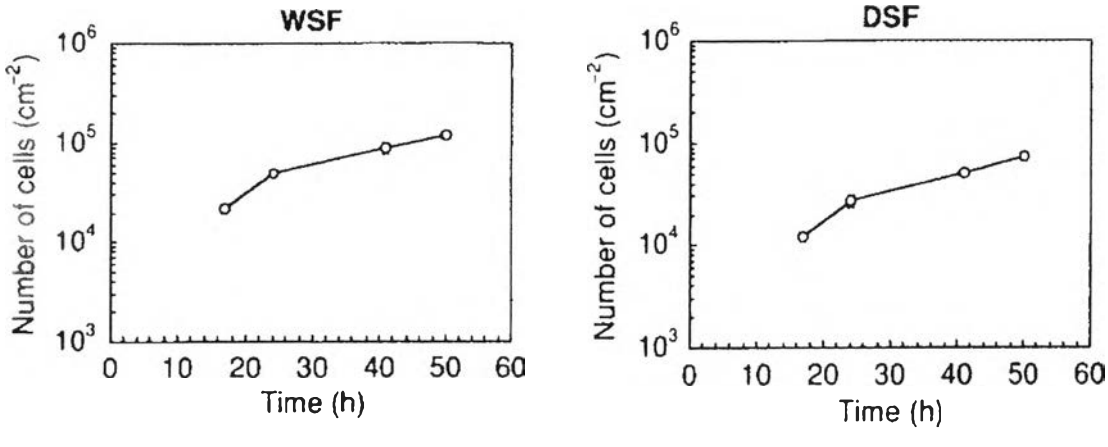
.15 FT-IR spectra of the as-prepared silk fibroin sponge (A), methanol-onges at various C/S ratios (B to E), and chitin whisker film (F). Arrows e absorption shoulder.

Baimark *et al.* (2010) studied biodegradable microparticles of (SF)/starch blends. The effect of SF/starch ratios on characteristics of the croparticles was investigated. From this study, the SF conformation of e matrices from FTIR analysis was changed from random coil to β-sheet ıding with starch. The amide I and II bands of SF shifted slightly to a number after blending with starch. This shifting increased when the blended ratio increased. These results indicated that the SF microsphere ıged from random coil to β-sheet form when the SF microsphere ended by starch.

2.4.2 SF as a Biomaterial

SF is the typical natural macromolecule spun by *Bombyx mori* silkworm that has been used as textile fiber and suture. In recent year, several researchers have investigated SF as one of the resources of biotechnology and biomedical materials because its unique properties including good biocompatibility, good oxygen and water vapor permeability, biodegradability, and minimal inflammatory reaction (Um *et al.*, 2001).

Minoura *et al.* (1995) studied the attachment and growth of fibroblast on matrices of silk fibroin. Silk fibroin from two different species of silkworm was studied. Both of cases showed attachment and growth of cells on matrices of silk fibroin for a long time. The typical cell growth curves on silk fibroin was shown in Figure 2.16. It can be concluded that silk fibroin is non-toxic to cells culture.



(a) (b)

Figure 2.16 Typical cell growth curves on silk fibroin from (a) *Bombyx Mori* domestic silkworm, (b) *Antheraea pernyi* wild silkworm.

Inouye *et al.* (1998) studied the growth of animal cells on silk fibroin-coated plates. Silk fibroin was used as the substratum for the culture of animal cells in place of collagen. It showed that silk fibroin enhances the growth of anchorage-dependent mammalian cells as the substratum, and the efficiency of silk fibroin is almost the same as that of collagen. Fibroin has no remarkable effects on the growth of anchorage-independent mammalian cells and insect cells.

Unger *et al.* (2004) investigated a novel biomaterial consisting of a non-woven fibroin net produced from silk (*Bombyx mori*) cocoons. Its ability can support the growth of human cells. The growth of cells on non-woven silk fibroin nets was illustrated in Figure 2.17. The cells including endothelial, epithelial, fibroblast, glial, keratinocyte, osteoblast cells were applied to the silk fibroin net and cultured for up to 7 weeks. All cases, the cells attached and spread onto the fibroin net. Therefore, silk fibroin nets are highly human cell-compatible and a useful new scaffolding biomaterial applicable for a wide range of target tissues in addition to supporting endothelial cells required for the vascularization of the newly formed tissue.

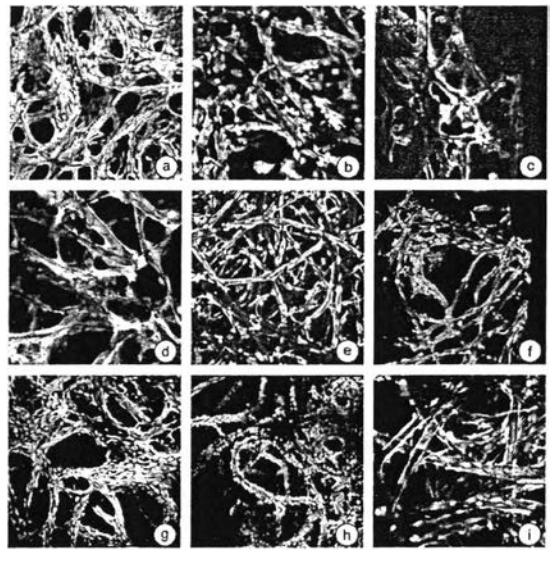


Figure 2.17 The growth of cells on non-woven silk fibroin nets. Cell name and (type) shown are: (a) 1321N1(glial); (b) A549 (epithelial); (c) CACO-2 (epithelial); (d) HaCaT (keratinocyte); (e) HPMEC ST1.6R (endothelial); (f) MRC-5 (fibroblast); (g) MG63(osteoblast); (h) STML-12 (epithelial); and (i) U373 MG (glial).

Wongpanit *et al.* (2007) studied the morphology of L929 fibroblast cells cultured on the methanol-treated silk fibroin and chitin whisker/silk fibroin sponges. After 6 hr of cultivation, the cells could attach on both types of sponges with their filopodia. These nanocomposite sponges exhibited the absence of the cytotoxicity as well as supporting cell spreading. Figure 2.18 illustrated SEM micrographs of attached cells on methanol-treated silk fibroin sponges

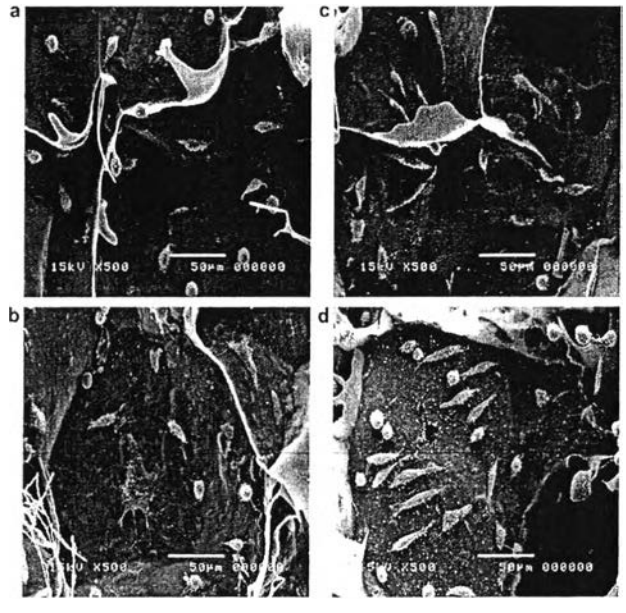


Figure 2.18 SEM micrographs of attached cells on methanol-treated silk fibroin sponges for (a) 6 and (b) 24 h of cultivation and SEM micrographs of attached cells on methanol-treated chitin whisker/silk fibroin sponges at C/S ratio of 4/8 for (c) 6 and (d) 24 h of cultivation.

2.5 Cellulose (CL)

Cellulose is one of the most abundant materials in the natural world that is used as a structural material primarily by both plants and some animals. It is a linear polysaccharide type of polymer with high molecular weight which is composed of linear chains of D-glucose linked by β-1, 4-glycosidic bonds. The chemical repeating unit of cellulose was shown in Figure 2.19. The degree of polymerization of native cellulose from various origins can fall in the range of 1000 to 30000 which corresponds to chain lengths from 500 to 15000 nm (Michael, 2008).

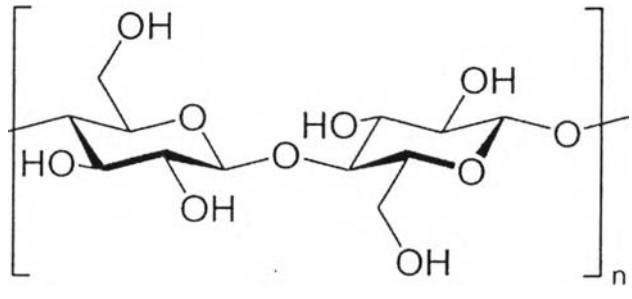


Figure 2.19 Chemical repeating unit of cellulose (www.en.wikipedia.org).

A cellulose fiber is composed of bundles of microfibrils where the cellulose chains are stabilized laterally by inter and intra molecular hydrogen bonding. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonds with oxygen atoms on neighbor chain. The chains hold together with side-by-side in order to forming microfibrils with high tensile strength. Microfibrils consist of simple fibrils where are linked by H-bonding. Generally, cellulose monocrystallite has been reported with length ranges from 100 to 300 nm and diameter between 5 and 20 nm. Cellulose monocrystallite has a high aspect ratio of 20-60 (Helbert *et al.*, 1996; Eichhorn *et al.*, 2001; Mathew and Dufresne, 2002; Samir *et al.*, 2004).

Under suitable conditions, transverse cleavage of the cellulose happens primarily in the amorphous zone of the fiber and releases needle-like monocrystals referred to cellulose whiskers. Whisker dimensions depend on both the origin of the cellulose and reaction conditions.

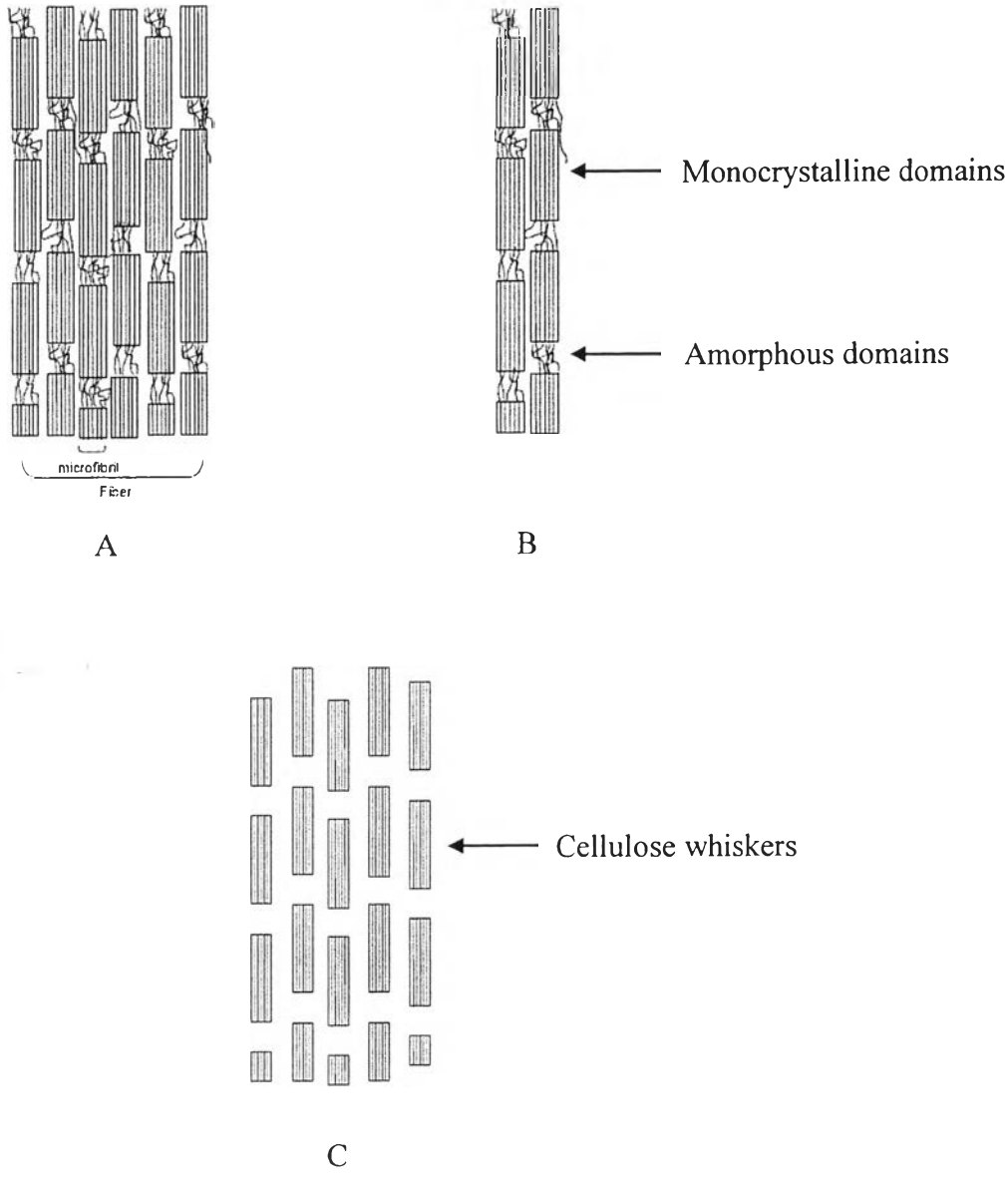


Figure 2.20 Cellulose fiber (A), Microfibrillated cellulose (B), Cellulose whiskers (C) (Senechal *et al.*, 2010).

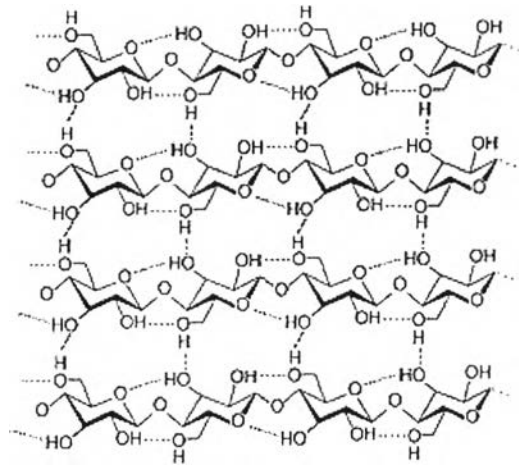


Figure 2.21 Inter and intra hydrogen bonding in CL (www.en.wikipedia.org).

Figure 2.20 shows model of cellulose fiber, microfibrillated cellulose and cellulose whiskers. Inter and intra hydrogen bonding in CL are shown in Figure 2.21. Because of the low cost, low density, good mechanical properties and recyclability of cellulose fiber, it is possible to use cellulose fiber as alternatives to synthetic fibers (glass or carbon) in composite applications. Furthermore, cellulose fibers are environmentally friendly, non-toxic and renewable materials. Therefore, manufacturing industries, especially packaging, building construction, automotive and furniture, have been encouraged to use plant fibers in their applications instead of the harmful and non-renewable reinforcing materials (Alamri *et al.*, 2012).

2.5.1 Cellulose Whiskers (CLWs)

Nanometer-sized high-purity single crystals of cellulose referred to cellulose whiskers, cellulose nanowhiskers or cellulose nanofibrils that can be obtained from various sources such as natural fibers, sea animals (Samir *et al.*, 2005). The extraction of CLWs from renewable sources has gained more attention in recent years due to their good mechanical properties (high specific strength and modulus), large specific surface area, high aspect ratio, environmental benefits and low cost (Orts *et al.*, 2005; Medeiros *et al.*, 2008).

The dimensions of CLWs are basically dependent not only on the acid species, acid concentration, time, and temperature of hydrolysis reaction, but also on

the different origins of cellulose (Azizi-Samir *et al.*, 2005). The rod-like CLWs particles from different sources common have a diameter and length in the range from 5 to 20 nm and 100 nm to several micrometers, respectively, after the acid hydrolysis (Candanedo, Roman, and Gray, 2005).

CLWs are also extracted by an acid hydrolysis which is removed the amorphous regions under certain process conditions. Sulfuric acid hydrolysis of cellulose is a well-known process used to remove amorphous regions. The use of sulfuric acid for CLWs preparation leads to more stable whiskers aqueous suspension than that prepared using hydrochloric acid. Actually, the H_2SO_4 prepared whiskers present a negative charge on surface, but the HCl prepared whiskers are not charged (Samir et al., 2005) (as shown in Figure 2.22). A number of negative charges on surface of CLWs contribute to a stable colloidal suspension due to the electrostatic repulsion (Lima and Borsali, 2004; Dufresne, 2006). Figure 2.23 illustrated suspensions of sugarcane bagasse whiskers.

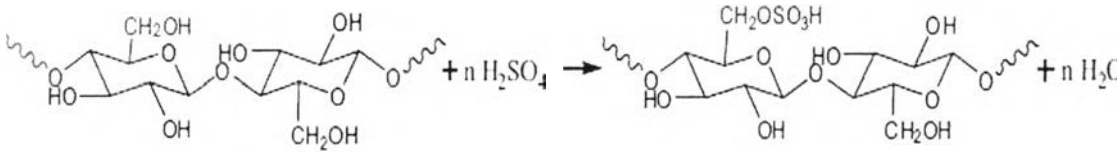


Figure 2.22 Esterification of cellulose hydroxyl groups during sufuric acid hydrolysis (Yang).

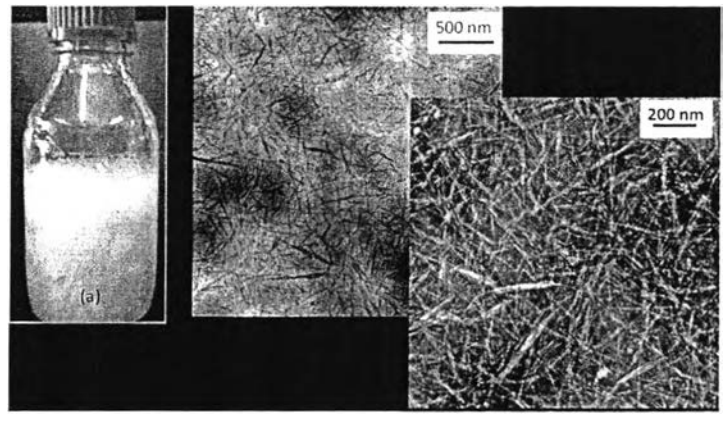


Figure 2.23 Suspensions of sugarcane bagasse whiskers were extracted at 30 min (Eliangela *et al.*, 2011).

Since many good properties of CLWs, the research on CLWs has been extensively developed in last decade. There are many researches that focusing on the use of CLWs extracted from cellulosic waste as filler. Agricultural wastes such as coconut husk fibers (Rosa *et al.*, 2010), cassava bagasse (Pasquini *et al.*, 2010), banana rachis (Zuluaga *et al.*, 2009), mulberry bark (Li *et al.*, 2009), soybean pods (Wang and Sain, 2007), wheat, straw, and soy hulls (Alemdar and Sain, 2008), and cornstalks (Reddy and Yang, 2005) have been studied as a resource in the production of CLWs.

Zuluaga *et al.* (2007) isolated cellulose microfibrils from the banana rachis by using chemical and mechanical treatments. Two different chemical treatments, peroxide/organosolv (PO) and peroxide/homogenization (PH) were evaluated. The width of individual cellulose microfrils closed to 5 nm in both treatments. The PO treated had a higher crystallinity because a more efficient removal of non-cellulosic, hemicelluloses, and lignin as well as dissolution of amorphous zones by the acid treatment.

Zuluaga *et al.* (2009) studied isolation of cellulose microfibrils from vascular bundles of banana rachis by using four different alkaline treatments. The cellulose microfibrils treated with peroxide alkaline, peroxide alkali-hydrochloric acid, 5 wt% and 18 wt% potassium hydroxide. They had average diameters of 3–5 nm, estimated lengths of several micrometers. TEM micrographs of negatively stained preparations of cellulose microfibrils isolated after different treatments were shown in Figure 2.24.

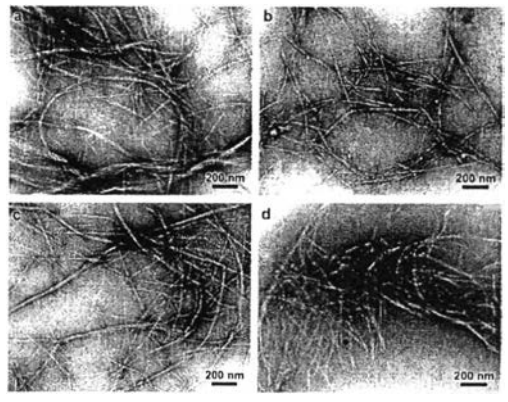


Figure 2.24 TEM micrographs of negatively stained preparations of cellulose microfibrils isolated after different treatments: (a) peroxide alkaline (PA), (b) peroxide alkaline–hydrochloric acid (PA–HCl), (c) potassium hydroxide 5 wt% (KOH-5) and (d) potassium hydroxide 18 wt% (KOH-18) (Zuluaga *et al.*, 2009).

Eliangela *et al.* (2011) extracted and characterized CLWs from sugarcane bagasse (SCB) as a source of cellulose. These CLWs were obtained after SCB underwent alkaline peroxide pre-treatment followed by acid hydrolysis at 45◦c for 30 minutes. CLWs were needle-like structures with an average length (L) of 255±55 nm and diameter (D) of 4±2 nm, giving an aspect ratio (L/D) around 64. They have good thermal stability (255◦c) and high crystallinity (87.5%).

Nurain *et al.* (2012) studied extraction and characterization of cellulose fibers and whiskers from rice husk. Fibers were obtained by treating rice husk with alkaline (NaOH) and following with bleaching. CLWs were extracted from these fibers by using sulphuric acid (H_2SO_4) treatment. TEM image from diluted suspension of CLWs extracted from rice husk fibers was illustrated in Figure 2.25. Most CLWs from rice husk displayed a diameter and aspect ratio in the range of 15-20 nm and 10-15, respectively.

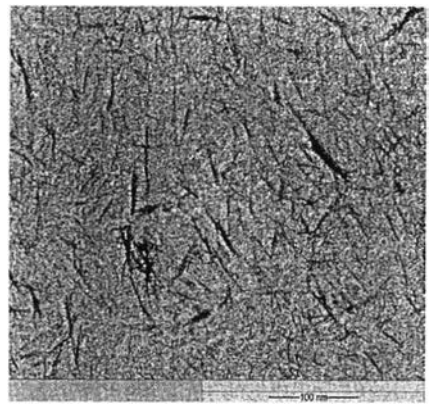


Figure 2.25 Transmission electron micrograph from diluted suspension of CLWs extracted from rice husk fibers (Nurain *et al.*, 2012).

CLWs have a great attention because their easy availability, easy modification chemical and mechanical, biocompatibility and renewability (Samir *et al.*, 2005). Therefore, CLWs are suitable for applications in polymeric matrixes that are acting as reinforcing elements.

Daniel *et al.* (2010) studied extraction of CLWs from cassava bagasse and their applications as reinforcing agent in natural rubber. Cellulose whiskers that have high aspect ratio were used to form nanocomposite film with a natural rubber matrix. A significant increase of the storage tensile modulus of film depended on CLWs filler.

Julien *et al.* (2010) isolated CLWs from bleached sugar cane bagasse kraft pulp in order to use as the reinforcing element in natural rubber nanocomposite to its enhance good properties. The effect of CLWs on tensile properties, thermal properties, moisture sorption, water vapor permeation, and soil biodegradation were studied. Incorporation of CLWs into rubber resulted in obtaining composites which has good thermal mechanical properties and biodegradability. Aspect ratio of CLWs is an important property in enhancing the tensile strength of nanocomposites. In addition, use of CLWs with hydrophobic polymer matrix such as rubber deteriorates its resistance to water vapor permeation.