CHAPTER II
LITERATURE REVIEW

2.1 Butanol

Butanol is a four carbon straight chained alcohol, colorless and flammable. Butanol can be blended with ethanol, ether and other organic solvent. Butanol can be used as a solvent, in cosmetics, hydraulic fluids, detergent formulations, drugs, antibiotics, hormones and vitamins, as a chemical intermediate in the production of butyl acrylate and methacrylate, and additionally as an extract agent in the manufacture of pharmaceuticals. The carbon atoms of butanol structure can form either a straight-chain or a branched structure, resulting in different properties. There exists different isomers, based on the location of the –OH and carbon chain structure (Liu *et al.*, 2013).

2.1.1 Properties of Butanol

Although the properties of butanol isomers are different in octane number, boiling point, viscosity, etc., the main applications are similar in some aspects, for example being used as solvents, industrial cleaners, or gasoline additives. All these butanol isomers can be produced from fossil fuels by many methods, only n-butanol, a straight-chain molecule structure can be produced from biomass. The different structures, properties and main applications are shown in Table 2.1

Table 2.1 Structures, properties and main applications of n-butanol, 2-Butanol, iso-Butanol and tert-Butanol (Liu *et al.*, 2013)

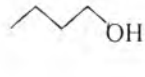
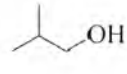
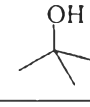
Properties	n-Butanol	2-Butanol	iso-Butanol	tert-Butanol
Molecular structure				
Density (g/cm^3)	0.81	0.806	0.802	0.789

Table 2.1 (cont.) Structures, properties and main applications of n-butanol, 2-Butanol, iso-Butanol and tert-Butanol (Liu *et al.*, 2013)

Properties	n-Butanol	2-Butanol	iso-Butanol	tert-Butanol
Boiling point(°C)	118	99.5	108	82.4
Melting point(°C)	-90	-115	-108	25.26
Refractive index(n20D)	1.399	1.3978	1.3959	1.3878
Flash point(°C)	35	22-27	28	11
Motor octane number	78	32	94	89
Main applications	Solvents-for paints, resins, dyes, etc. Plasticizers-improve a plastic material processes Chemical intermediate -for butyl esters or butyl ethers, etc. Cosmetics-including eye makeup, lipsticks, etc. Gasoline additive	Solvent Chemical intermediate-for butanone, etc. Industrial cleaners - paint removers Perfumes or in artificial flavors	Solvent and additive for paint Gasoline additive Industrial cleaners -paint removers Ink ingredient	Solvent Denaturant for ethanol Industrial cleaners- paint removers Gasoline additive for octane booster and oxygenate Intermediate for MTBE, ETBE, TBHP, etc.

2.1.2 Butanol as Fuel

Not only the use of solvent, chemical intermediate and extract agent but butanol also can be used as fuel, which attracted people's attention in recent years. Butanol in particular appears to be a good choice as a fuel additive to reduce exhaust smoke and particulates (Ladisch, 1991). Butanol has good properties of high heat value, high viscosity, low volatility, high hydrophobicity, less corrosive, butanol has the potential to be a good fuel in the future. The properties of butanol and other fuels or homologues are compared in Table 2.2.

Table 2.2 Properties of butanol and other fuels (Freeman *et al.*, 1988; Dean and Lange, 1992)

Fuel	Octane no.	Cetane no.	Evaporation heat (MJ/kg)	Combustion energy (MJ/dm^3)	Flammability limits (%vol)	Saturation pressure (kPa) at 38°C
Gasoline	80-99	0-10	0.36	32	0.6-0.8	31.10
Methanol	111	3	1.2	16	6-36.5	31.69
Ethanol	108	8	0.92	19.6	4.3-19	13.8
Butanol	96	25	0.43	29.2	1.4-11.2	2.27

Butanol demonstated the good properties compared with its homologues such as 2-butanol, iso-butanol, and tert-butanol and other fuels such as gasoline and ethanol. Actually, when ethanol is blended with gasoline (less than 10%), there exists some disadvantages. Firstly, the heating value of ethanol is one sixth of gasoline. The fuel consumption will increase 5% if the engine is not retrofitted. Secondly, acetic acid will be produced during the burning process of ethanol, which is corrosive to the materials of vehicle. The preservative must be added when the ethanol proportion upper than 15%. Thirdly, ethanol is hydroscopic and the liquid phase separation may be occurring with high water proportion.

Furthermore, ethanol as fuel cannot be preserved easily and it is more difficult in the process of allocation, storage, transition than that of gasoline.

Butanol has a lot of characteristics that make it a better fuel extender than ethanol, now used in the formulation of gasohol. It can solve many problems associated with the use of ethanol. This is advantages of butanol over ethanol (Huang *et al.*, 2004); 25% more British thermal units per gallon, less evaporative/explosive with a Reid vapor pressure 7.5 times lower than ethanol, safer than ethanol because of its higher flash point and lower vapor pressure, and more miscible with gasoline and diesel fuel but less miscible with water.

2.2 Biobutanol

Butanol can be produced in the process of fermentation by bacteria and butanol as one of the products called biobutanol. The most popular bacteria species used for fermentation is *Clostridium acetobutylicum*. Because the main products of this process containing acetone, butanol, and ethanol, the fermentation is called ABE fermentation (Qureshi *et al.*, 1992).

2.2.1 Biological Process

Not only the chemical process, but butanol can also be obtained from biological process with the renewable resources by the microorganism through fermentation. The *Clostridia genus* is very usual for butanol synthesis under anaerobic conditions, and the fermentation products are often the mixture of acetone, butanol, and ethanol. A few kinds of *Clostridium* can utilize cellulose and hemicellulose with the ability of cellulolytic activities (Mitchell, 1997; Berezina *et al.*, 2009).

Compared with the chemical process for butanol production, biological process has the distinct advantages. For example, it can utilize the renewable resources such as wheat straw, corn core, switch grass, etc. Furthermore, biological process has high product selectivity, high security, less by-products. Moreover, the fermentation condition of butanol production is milder than that of

chemical ways and the products are easier to separate. The process of biobutanol production with Lignocellulosic feedstocks is as follows:

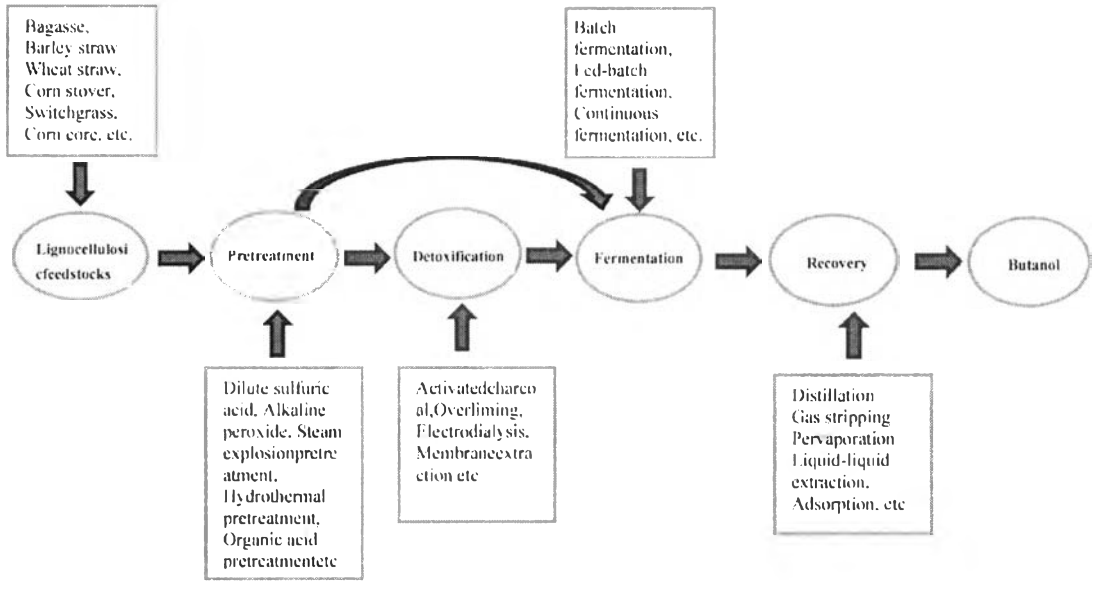


Figure 2.1 Butanol production process from lignocellulosic feedstocks (Liu *et al.*, 2013).

2.3 Acetone-Butanol-Ethanol (ABE) Fermentation

The production of acetone, butanol, and ethanol (ABE) by fermentation is a process that had been used by industry for decades. The bioproduction of acetone, butanol, and ethanol (ABE) by solventogenic *Clostridia*, such as *Clostridium acetobutylicum*, was once the second largest biotechnological industry in the world (Jones and Woods, 1986) and has attracted renewed interest for many economic and environmental reasons in recent years (Liu *et al.*, 2013).

Acetone, butanol, and ethanol are typically produced in an approximate ratio of 3:6:1 (w/w). Butanol, an important industrial chemical and excellent alternative to gasoline, is the preferred solvent and attracts the highest price (Green, 2011; Jang *et al.*, 2012). Batch production remains the conventional method for ABE

fermentation, although the productivity of a traditional ABE batch has been mostly in the range of 0.1–0.3 g/l/h (Qureshi *et al.*, 2000).

2.3.1 Microbes

Clostridium is a group of obligate, Gram positive, endospore-forming anaerobes. *Clostridium* while most of members of this genus are strict anaerobes, some may grow in the presence of oxygen. Catalase is not usually produced (Benson, 2001). There are lots of strains used for ABE fermentation in different culture collections, such as ATCC (American Type Culture Collection), DSM (German Collection of Microorganisms, or Deutsche Sammlung Von Mikroorganismen), NCIMB (National Collections of Industrial & Marine Bactria Ltd), and NRRL (Midwest Area National Center for Agriculture Utilization Research, US Department of Agriculture). The different strains share similar phonotype such as main metabolic pathway and end products. Molecular biology technology offers efficient method for classification. The butanol-producing *Clostridium* can be assigned to four groups according to their genetic background, named *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutyl acetonicum*, and *C. saccharobutylicum*, respectively. *C. acetobutylicum* is phylogenetically distinct from the other three groups (Liu *et al.*, 2013).

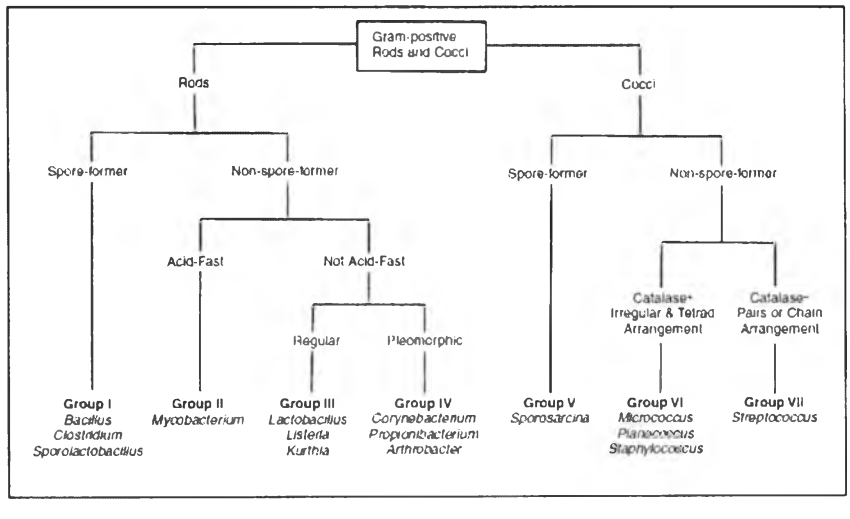


Figure 2.2 Separation outline for gram-negative rods and cocci (Benson, 2001).

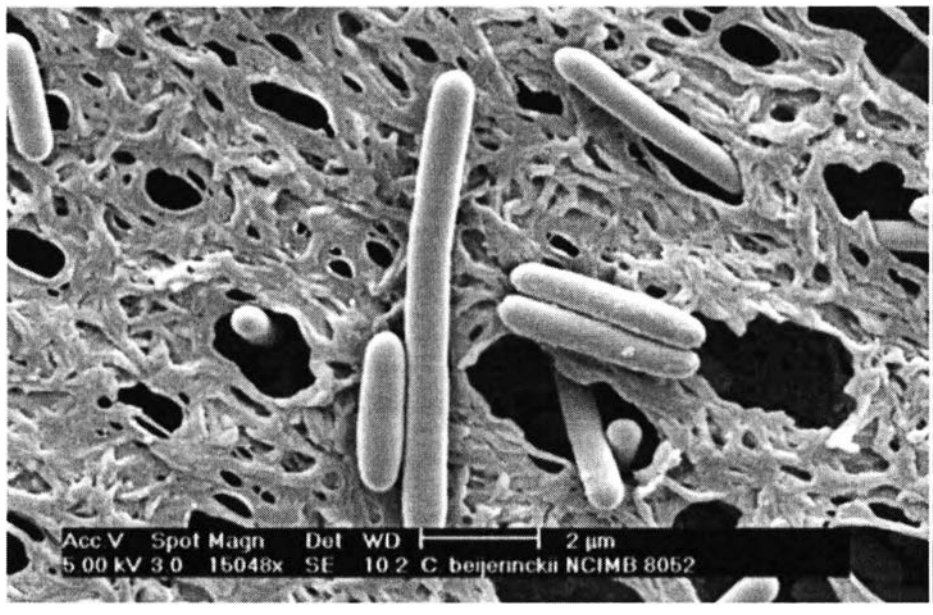


Figure 2.3 SEM of *Clostridium beijerinckii* NCIMB 8052 (Grigoriev *et al.*, 2011).

2.3.2 Culture Medium

A microbiological medium is the food that use for culturing bacteria, molds, and other microorganisms. It can exist in three consistencies: liquid, solid, and semisolid. For construct a medium that achieve a desired result in the growth of organisms, the medium must have nutrients need of bacteria and suitable factors that are: water (distilled water), carbon source (carbon dioxide or organic source of carbon), energy (e.g. glucose, amino acid, nitrite, nitrate, and sulfide), nitrogen (e.g. amino acid, beef extract, and peptone), minerals (such as sodium, potassium, calcium, magnesium, manganese, iron, zinc, copper, phosphorus, and cobalt), growth factor (certain amino acids or vitamins), and pH of the medium (suitable pH for bacteria grow is about pH 7 or slightly lower so the pH of nutrient broth should be adjusted to pH 6.8). (Benson, 2001)

CMM or Cook Meat Medium is used for cultivation of both aerobic and anaerobic microorganisms (Holman, 1919), especially pathogenic *Clostridia.* This can also be used as a maintenance medium for stock cultures. CMM's composition are consist of beef heart 98 g/l, proteose peptone 20 g/l, dextrose 2 g/l, and sodium chloride 5 g/l (HiMedia, 2011). Beef heart and proteos peptone are nitrogen source that provide amino acids and other nutrients. Dextrose's addition

allows rapid and heavy growth of anaerobic microorganism in a short period and leads to more rapid identification of important anaerobes. CMM's formulation supports the growth of many spore forming and non-spore forming strict anaerobes. It has the potential to initiate growth of bacteria from very small inocula and to maintain the viability of cultures over long period of time. There showed the survival of bacteria mixed culture in CMM without displacing the slower-growing organisms. The products of growth do not rapidly destroy the inoculated organisms and thus it is an appropriate medium for the storage of aerobic and anaerobic organisms. Therefore CMM is used for cultivation and maintenance of *Clostridia* and for determining proteolytic activity of anaerobes.

A *Clostridia* culture was used to inoculate P2 medium containing: carbon and nitrogen source (glucose 60 g/l, yeast extract 1 g/l), acetate buffer (KH_2PO_4 0.5 g/l, K_2HPO_4 0.5 g/l, ammonium acetate 2.2), vitamins (para-amino-benzoic acid 0.001 g/l, thiamine 0.001 g/l, biotin 0.00001 g/l), and minerals ($MgSO_4 \cdot 7H_2O$ 0.2 g/l, $MnSO_4 \cdot 7H_2O$ 0.01 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, NaCl 0.01 g/l). The glucose and yeast extract solution was sterilized by autoclave at 121°C and cool down to room temperature. Stock solutions of buffer, vitamin, and mineral solutions were filtrated by sterilized filter 0.2 μm and added to the glucose and yeast extract solution to the above concentration (Qureshi and Blaschek, 1999).

2.3.3 Metabolic Pathway

The obligate anaerobe, *C. acetobutylicum*, has long been of great interest due to its ability to ferment several substrates into valuable final products such as acetone, butanol, and ethanol. Fermentation by this organism is characterized by two distinct phases. In the first phase, growth is rapid with copious hydrogen evolution and acetic and butyric acid production. As a result, the pH decreases to a characteristically low value (typically, about 4.5). This is followed by a second, slower growth phase with decreased hydrogen evolution and solvent formation, with partial uptake of the acids that were formed during the first growth phase. The two phases are termed acidogenic and solventogenic, respectively (Rao and Mutharasan, 1987). Determination of whether *Clostridium* utilizes a solvent or acidic producing pathway is dependant on ATP and NADH levels (Mathews and Wang, 2009).

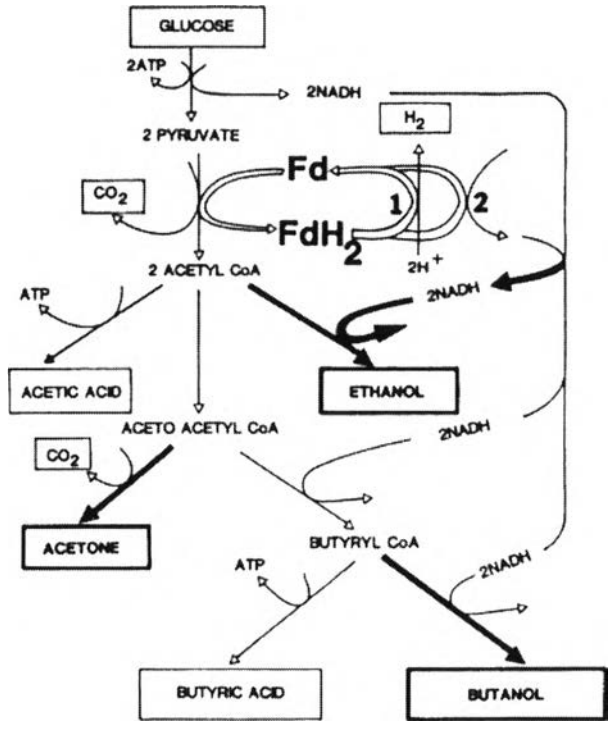


Figure 2.4 Metabolic pathway of *C. acetobutylicum*. The following enzymes are shown: hydrogenase (1), NAD-linked Fd oxidoreductase (2). CoA, Coenzyme A. (Rao and Mutharasan, 1987).

The metabolic pathway of *C. acetobutylicum* is shown in Figure 2.4. Glucose is fermented via the EMP (Embden-Meyerhoff-Parnas) pathway to pyruvate. Pyruvate is oxidized to acetyl coenzyme. A requires obligatory ferredoxin (Fd) reduction. Reduced Fd (FdH_2) is oxidized by hydrogenase, which regenerates Fd and releases electrons as molecular hydrogen. Furthermore to formation by pyruvate oxidation, FdH_2 may be formed by NADH oxidation. This permits the regeneration of NAD^+. The activity of NAD-linked Fd oxidoreductase control this reaction (Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976). The reaction is reversible, and depending on culture conditions, either FdH_2 or NADH may be formed. It should be noted that the reverse reaction (NADH formation from FdH_2) is inhibited by NADH and proceeds only on rapid removal of the NADH that is formed (Petitdemange *et al.*, 1976). The mechanism of NADH removal in vivo is alcohol

formation (Figure 2.4). It is a mechanism that we have sought to use to redirect carbon flow toward the alcohols.

2.3.4 Immobilization

The traditional ABE fermentation has many disadvantages for example low solvent yield and productivity, problem of toxic effects that butanol has on microorganisms and unstable for solvent production (Qureshi *et al.*, 2000). Hence to enhance ABE fermentation, the cell immobilization has gained much attention. Several attempts have been made to examine the potential applications for a cell immobilization technique, either through entrapment or by adsorption, aimed at increasing butanol productivity (Yen *et al.*, 2011).

The immobilization of desired bacteria onto suitable materials as carriers is currently gaining much attention in various fields of biotechnology. By the process of immobilization of bacteria a higher cell density in bioreactors can be achieved and based on this, smaller reactors, shorter retention time or higher flow rates can be employed (Hrenovic *et al.*, 2011).

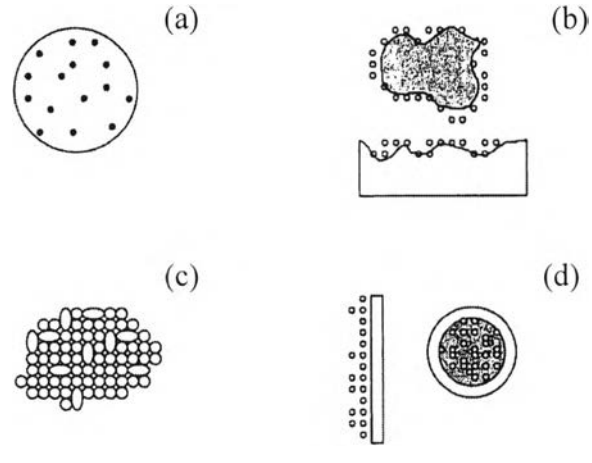


Figure 2.5 Basic methods of cells immobilization: (a) Entrapment with a matrix, (b) Attachment or adsorption to a preformed carrier, (c) self-aggregation of cells and (d) cells contained behind a barrier (Pilkington *et al.*, 1998).

The immobilized cells system is any systems in which microorganism are confined within a bioreactor, so allowing their reuse. Normally, there are four methods of immobilization techniques can be classified, depended on the cell localization's physical mechanisms. These methods are entrapment with in porous matrix attachment or adsorption to preformed carrier, self-aggregation or crosslinking agents, and cells contained behind a barrier (Pilkington *et al.*, 1998).

The reduction of costs involved in ethanol production from bioprocesses employing immobilized cells systems are associated with aspects such as the cost of raw materials, the use of less expensive, abundant and stable immobilization adjuncts, the high cell concentration in the bioreactors, the simplicity and low cost of immobilization techniques, the stability of the immobilized biocatalyst in its operational state, ease of regeneration and the design and development of a suitable bioreactor system (Kourkoutas *et al.*, 2004; Wang *et al.*, 2011).

The advantages of this method are simplicity, minor influence on conformation of the biocatalyst and there is no need for utilization of chemicals which could cause a damage of bacterial cells, so the catalytic activity could be preserved. From the aspect of lactic acid fermentation (Djukić-Vuković *et al.*, 2013) immobilization has several advantages such as higher cell density, better cell stability and it enables easy separation and re-circulation of bacterial biomass.

Moreover, immobilization of cells for use in continuous culture fermentations has several advantages over batch cultures that are commonly used to perform fermentation. Immobilized cell systems are able to maintain high cell concentrations, generally have improved reaction rates, and are stable at high dilution rates with little cell washout and simplicity of operation (Welsh *et al.*, 1987). Other advantages are that the reactor configuration can be relatively simple, and support structure can often be reused (Krouwel *et al.*, 1983). No chemical addition is necessary to achieve cell immobilization on this support (Qureshi *et al.*, 2000).

The ideal carrier of bacteria should be inert, nontoxic, of porous structure, relatively cheap, easily available, environmental friendly and should provide a rough, irregular surface available for colonization (Durham *et al.*, 1994). On such types of carriers the whole external surface, cavities, cervices, irregularities

and all pores with diameter of approximately greater than or equal to 3 μm are accessible for dense bacterial colonization (Hrenovic *et al.*, 2011).

A significant amount of research work has been done on ABE production using immobilized cell continuous culture systems, which improve reactor productivity and result in more stable reactors. These systems have examined the use of immobilized cells by two main techniques, adsorption and entrapment. Examples of cell immobilization by entrapment include carrageenan and chitosan (Frick and Schugerl, 1986), calcium alginate (Häggström and Molin, 1980; Krouwel *et al.*, 1980; Schoutens *et al.*, 1985), while that of adsorption include coke (Welsh *et al.*, 1987), beechwood shavings (Forberg and Haggstrom, 1985), and bonechar (Qureshi and Maddox, 1987). Bonechar immobilized cells of *Clostridium acetobutylicum* demonstated that this type of system has great potential for high solvent productivity and stable reactor operation (Qureshi and Maddox, 1987). These reactor systems have simple operation, can be operated for long periods, and are relatively low cost reactors.

Table 2.3 Comparison of continuous reactor performance with different cell immobilization techniques (Qureshi *et al.*, 2000; Zhang *et al.*, 2009)

Immobilized cell system	**Total solvent (g/l)**	**Productivity (g/l/h)**	**Yield (g/g)**	**Reference**
Free cell	9.4	0.22	0.24	(Lee *et al.*, 2008)
Polyvinyl alcohol	22.1	0.4	0.44	(Lee *et al.*, 2008)
Brick	7.9	15.8	0.38	(Qureshi *et al.*, 2000)
Bonechar	6.5	6.5	0.38	(Qureshi and Maddox, 1988)
Cotton towel	8.5	7.6	0.53	(Huang *et al.*, 2004)

Table 2.3 (cont.) Comparison of continuous reactor performance with different cell immobilization techniques (Qureshi *et al.*, 2000; Zhang *et al.*, 2009)

Immobilized cell system	Total solvent (g/l)	Productivity (g/l/h)	Yield (g/g)	Reference
Carragenan	4	2.8	0.18	(Frick and Schugerl, 1986)
Chitosan	2.7	1.43	0.18	(Frick and Schugerl, 1986)
Corn stalk	5.1	5.06	0.32	(Zhang *et al.*, 2009)
Coke	12	1.12	0.3	(Welsh *et al.*, 1987)
Beechwood	3.8	1.53	-	(Forberg and Haggstrom, 1985)
Ca alginate	4	4	0.18	(Frick and Schugerl, 1986)
Sponge	15.5	4.2	0.43	(Park *et al.*, 1989)

From the previous study, Qureshi *et al.* (2000) examined solvent productivity of *C. beijerinckii BA101* when immobilized by adsorption in a simple continuous packedbed reactor. Adsorption is a simple technique and results in superior reactor productivities than those immobilized by entrapment (Qureshi and Maddox, 1987). The substance chosen to immobilize cells was clay brick because it is readily available, and an inexpensive material. The process of immobilization was found to be relatively simple, consisting of adding actively growing culture to the column containing the brick support, and allowing the cells to adhere and grow.

ABE fermentation has long been subjected to a strong final product inhibition (mainly due to butanol), which has adversely affected the economics of its commercial production. The primary limitation associated with ABE fermentation are the toxic effects that butanol has on microorganisms, leading to low solvent productivity. Therefore, it was necessary to search for high butanol tolerant microorganism strains as well as to employ in situ toxicity removal technologies (such as stripping, adsorption, and pervaporation) in order to overcome the toxicity problem.

From Yen *et al.* (2011)'s study, three common carriers; bricks, synthetic sponge, and nonwoven fabric were aimed to determine an optimal carrier for the immobilization of *Clostridium acetobutylicum* in ABE fermentation. The optimal size and number of carriers used for cell immobilization in butanol production was also investigated. The production performance of ABE as well as the process stability for a repeated batch operation of the optimal carrier using immobilized cells was also discussed in this study. The reason of using brick in this study is the high density of the bricks was easier to precipitate in the reactor (density of 1.8–2.0 g/cm^3). This may be convenient when using the repeated batch format and the continuous operation format. In addition to a simple operation and easy availability, the low price of bricks was another of its advantage as an immobilization carrier (Qureshi *et al.*, 2004).

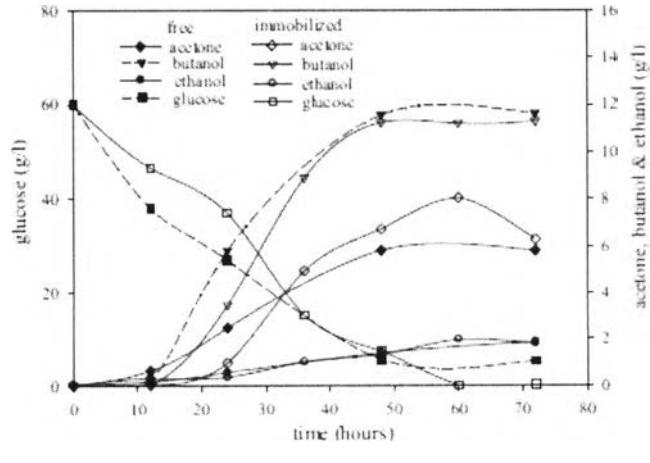


Figure 2.6 Comparison of glucose consumption and butanol production between the batch with free cells and cells immobilized on bricks (Yen *et al.*, 2011).

From the experiment indicates that the possible limitation of nutrient transfer in the batch on bricks with immobilized cells did not impede ABE fermentation. Both batches indicated similar butanol productivity (Figure 2.6).

It is clear that the cells were adsorbed onto the brick surfaces, as shown in Figure 2.7. However, due to technical difficulties, there was no way to measure how many cells were adsorbed either on the surfaces or inside the bricks. Therefore, they could only demonstrate that the addition of bricks using sugar glucose as the carbon source would not obstruct ABE fermentation at this stage.

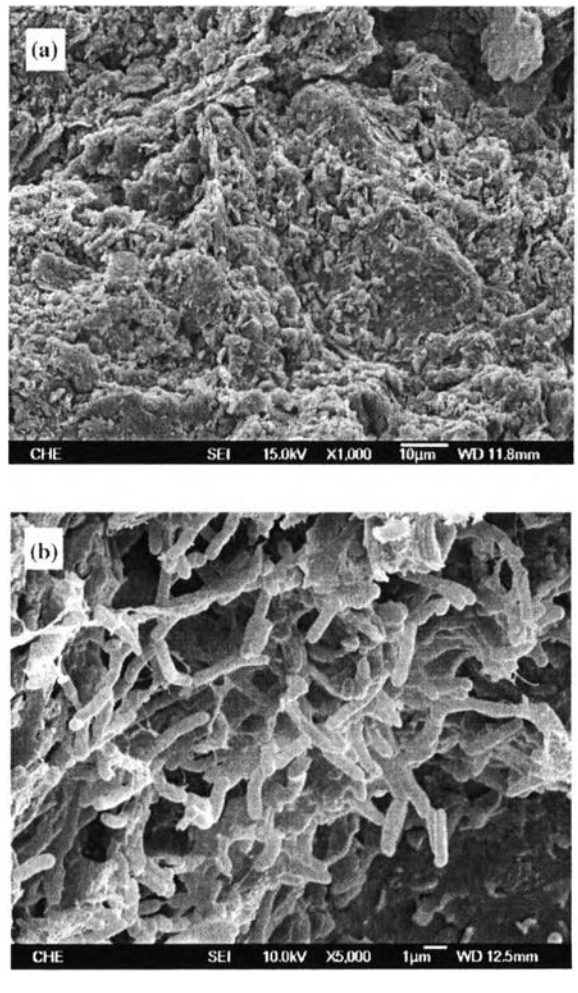


Figure 2.7 Scanning electron micrographs of immobilized cells on bricks: (a) before the immobilization and (b) after the immobilization (Yen *et al.*, 2011).

Moreover, the result of this study demonstrated that small particles of brick (0.15–2.4 mm) may have led to improved ABE fermentation in comparison with the other two batches containing medium and large sized brick pieces. The result indicates that cell immobilization on bricks produced a stable solvent production. This can be explained by the successfully repeated batch operation demonstrated that the cells were adsorbed not only on the outside surface of the bricks but also on the inside. Therefore, a long term operation using continuous feeding nutrients might be a valid way to enhance ABE productivity. After 6 cycles of the operation, no decline in solvent production was observed in the repeated batch operation using cell immobilization onto bricks.

The ease of the immobilization process along with the economic considerations derived from both times and labor savings, makes using bricks as carriers for ABE fermentation very attractive. The SEM pictures indicated that the cells had become attached to the surface of the brick through adsorption. No nutrient transfer limitation was observed in the operation using cells immobilization on bricks.

The results from previous study indicated that dry Ca-alginate is a well matrix system that almost doubles the productivity of a free cell batch (Frick and Schugerl, 1986). The application of calcium alginate in a continuous butanol fermentation derived from whey permeate has also been studied. The productivity was sixteen times higher than that obtained from a free cell batch (Largier *et al.*, 1985; Schoutens *et al.*, 1985).

One of the most extensively used classes of natural support is lignocellulosic materials. Some lignocellulosic supports being investigated include sawdust, wood chips/shavings, rice husks, cotton towels, and straw. These advantages include being highly porous as well as having a good water retention capacity. One of the most attractive properties of using cellulosic materials as an immobilization support is that the spent materials can be sent back to the hydrolysis process for sugar production, thus minimizing waste generation (Zhang *et al.*, 2009). Many researchers (Shukla *et al.*, 1989; Huang *et al.*, 2004) found that *Clostridium* can automatically stick to the lignocellulosic material and grow on the support without any additional chemical.

Butanol is a very hydrophobic compound. It is toxic and has been shown to disrupt membrane-linked functions, lower intracellular ATP levels, and inhibit sugar uptake (Alsaker *et al.*, 2010). The inhibitory threshold of wild *Clostridium* is generally considered to be about 13 g/l and the growth of *C. acetobutylicum ATCC824* would be completely inhibited at a butanol concentration of 14 g/l (Ounine *et al.*, 1985; Nielsen and Prather, 2009). Therefore, most wild *C. acetobutylicum* strains only produce about 10–13 g/l butanol at an initial glucose concentration of 60 g/l. Since low butanol titers result in high recovery costs, some chemically mutated or genetically engineered *C. acetobutylicum* strains have been created to improve their butanol tolerance.

To further overcome the problems of low butanol titer and productivity, cells were immobilized onto a fibrous matrix (cotton towel) by surface adsorption to improve their butanol tolerance and applied to biobutanol production in a repeated batch fermentation model (Liu *et al.*, 2013).

Dispersed cells can come together and form polymicrobial aggregates attached to a solid surface. This cell-solid surface contact has been recognized as providing significant structure modulations and phenotype changes in the anchored cells, one of which is an increased resistance to antimicrobial agents (Mah and O'Toole, 2001). From previous study (Liu *et al.*, 2013), a fibrous matrix (cotton towel) was applied to immobilize *C. acetobutylicum B3* cells by surface adsorption. It seems that the cells tended to propagate on the fibrous matrix rather than in the bulk medium, as indicated by the low turbidity of the culture with cotton towel. SEM images demonstrated that the surface adsorbed cells were located in aggregates and effectively immobilized by the extracellular polymeric substances (EPSs) they produced. Unlike the aggregates of surface adsorbed cells, the morphology of the planktonic cells showed that the cells grew separately from each other, free of EPS cover.

From the characterized for butanol tolerance of the *C. acetobutylicum* B3 cells immobilized on the fibrous matrix demonstrated that the fibrous matrix significantly improved the butanol resistance of the growing cells. The survival of the immobilized cells exposed to high butanol concentrations for 2 hours was dramatically enhanced, and was about 3 orders of magnitude higher than that of the

planktonic cells (free mobilized cells). Thus, the immobilization of *C. acetobutylicum* B3 by adsorption to the fibrous matrix rendered the cells more resistant to butanol.

This is because when cells attach to a solid surface, they exhibit a different pattern of gene expression. Some genes are repressed or activated, and the cellular structure (e.g. plasma membrane composition) is modulated, leading to a resistant phenotype (Flemming and Wingender, 2010). When *C. acetobutylicum* B3 cells were detached from the fibrous matrix, their survival rate was lower than that of the EPS-immobilized cells after exposure to butanol, but still much higher than that of the planktonic cells (free mobilized cells), suggesting the extracellular polymeric substance (EPS) produced by the cells when attached to the fibrous matrix could also confer resistance. The EPS plays an important role in exclusion of toxic substances and maintenance of a highly hydrated microenvironment (Flemming and Wingender, 2010). Since butanol is a very hydrophobic compound, the hydrated microenvironment maintained by the EPS may be of especial importance for ability of the cells to resist butanol toxicity.

Zeolites are aluminosilicates with well-defined crystalline structures that contain aluminium, silicium and oxygen in their regulatory framework. The nanoporous crystal structure retains cations, water and has shown a great capacity for ammonia nitrogen (NH_4-N) and heavy metal adsorption (e.g. Cu, Cd, Pb, and Zn), thus removing molecules toxic to microorganisms in anaerobic and aerobic digestion processes (Green *et al.*, 1996; Tada *et al.*, 2005). Moreover, zeolites can be purpose fully modified by loading trace metal elements (Fe, Mg, Ni and Co) in variable concentrations on their surface which favours methanogenic and acidogenic bacteria to be grouped in small micro-colonies, supplying co-factors for enzyme biosynthesis. Consequently, zeolites are used for the immobilization of microorganisms in anaerobic reactors to stabilise and optimize the process efficiency (Milán *et al.*, 2003). Zeolite was used for immobilizing hemicellulolytic bacteria populations on zeolite leads to an increase of methane yields in batch-culture experiments (Weiß *et al.*, 2010).

From previous studies (Bauman *et al.*, 2001; Hrenovic *et al.*, 2009), among the inorganic carriers, natural zeolite tuffs (NZ) have been shown as a good

material for the immobilization of desired yeast and bacteria. Naturally zeolites often occur in tuffs rich in more than 48 known zeolite mineral species such as analcime, chabazite, clinoptilolite, erionite, laumontite, mordenite, phillipsite, stilbite. The natural clinoptilolite was usually chosen in studies on the wastewater treatment on the base of its widespread occurrence in the nature, price-easily accessibility and feasibility, inexpensive, large surface area, rigidity, surface functionality, thermal, mechanical and radiation stability (Hrenovic *et al.*, 2011).

Zheng *et al.* (2012) studied the alginate-based immobilized yeast with zeolite that include MCM-41 exhibited much shorter fermentation time and higher ethanol concentration than pure alginate. The reason for the higher bio-catalytical function of the immobilized yeast might lay in the uniformly yeast distribution in the bio-reactor and high yeast cell concentration, which contributed by the improved transmission of fermentation media and combined effects of yeast adsorption by MCM-41 and embedment by alginate. To increase ethanol productivity and to decrease labor intensity, aspects such as bioreactor volume, energy consumption in the production of ethanol, and cell immobilization for ethanol production have been extensively studied and reviewed. The preparation of organic–inorganic composite materials for yeast immobilization appears very attractive as a prospective method because it combines features of organic and inorganic materials. These organic–inorganic carriers not only utilize their good embedded affinity to organic materials and fast propagation of yeast cells, but also possess good mechanical properties and permeability to inorganic materials; these traits prolong the operating life of carriers, and effectively improve the fermentation efficiency of immobilized yeast in production of ethanol as a fuel.

The alginate-based immobilized yeast with zeolite that include MCM-41 has the highest biology capacity of 4.8 x 10^9 cells/ml, and can be utilized repeatedly in batch and continuous fermentation applications. The published literature (Chen *et al.*, 2007) reported that mesopores and macropores as being among carriers those are vital to the high density proliferation of the yeast as well as transportation of substances and products between carriers and the medium. In the study of Zheng *et al.* (2012), yeast cells were immobilized with the carriers modified by various inorganic fillers. Composite carriers with three microporous inorganic

fillers, i.e. H_2NZSM-5, H_2NH-b, and H_2NH-Y, were observed to get low cell densities, and the fermentation time was relatively long with these microporous inorganic fillers. The other three mesoporous inorganic fillers, i.e. a- AlOOH, c-Al_2O_3, and H_2NMCM-41, composite carriers were found to achieve high cell densities for immobilizing yeast cells.

However, a major disadvantage of immobilization technique is the relative weakness of the adsorptive binding forces (Panesar *et al.*, 2007). From Djukić-Vuković *et al.* (2013)'s study the *Lactobacillus rhamnosus* were immobilized onto zeolite, a microporous aluminosilicate mineral and the lactic acid production with free and immobilized cells was compared. The powdered zeolite molecular sieves 13X were used for immobilization of *Lactobacillus rhamnosus*. Zeolites are crystalline, highly porous aluminosilicate minerals. Type X has a basic structure with exchangeable sodium ion.

The result from this study demonstrated that *L. rhamnosus* ATCC 7469 strain produces exopolysaccharides (EPS) which formed a dense, sticky layer on the surface of bacterial cells. It is possible, although not yet experimentally proved that the EPS could contribute to better attachment of the cells to the zeolite carrier. It is previously shown that zeolite surface could adsorb biopolymers like proteins and nucleic acids (Kubota *et al.*, 2008). Physiological role of bacterial EPS is still unknown, although due to their variability in composition and physical characteristics different functions were proposed. EPS could have protective role against harsh environmental conditions, act as adhesives for interactions with other surfaces or substrates, and promote bacterial aggregation and formation of bio-film (Badel *et al.*, 2011). From the report of Weiß *et al.* (2011), colonization of zeolite for anaerobic fermentations and biogas production, in this study, *L. rhamnosus* cells were rather tightly attached to zeolite support. The strong bond of *L. rhamnosus* cells with zeolite surface could be illustrated with the time required for desorption of cells from the carrier. In the study of (Hrenovic *et al.*, 2009) detachment of cells was complete after 3 minutes of vigorous shaking at 40 Hz. In this study, for the detachment of immobilized *L. rhamnosus* cells from zeolite particles 10 minutes of shaking was necessary.

The strong bond between *L. rhamnosus* and zeolite surface could be a result of electrostatic interaction between negatively charged cell surface of *L. rhamnosus* ATCC 7469 strain and positively charged zeolite surface. It is documented that the cell surface of *L. rhamnosus* ATCC 7469 is negatively charged in the wide range of pH values and it acts as a Lewis base (Pelletier *et al.*, 1997). The zeolites are well known as Lewis acids (Miura *et al.*, 2009) which could enhance binding of *L. rhamnosus* cells with zeolite surface.

The productivity achieved in the first fermentation cycle with immobilized cells was almost threefold higher than the productivity obtained in free cell system. It can be seen that both the sugar consumption and lactic acid production were faster in immobilized system comparing to the batch fermentation with free bacteria.

The higher lactic acid productivity and better survival of bacterial cells in immobilized system is most probably because combined effect of zeolite buffering capacity and increased productivity of the bacteria in a biofilm on zeolite particles. Because of the advantages that could be achieved, a significant number of papers are dealing with immobilization of lactic acid bacteria as a good strategy for enhancement of the process productivity (Djukić-Vuković *et al.*, 2013). Average productivity of the process with immobilized cells was 1.32 g/l/h which is significantly higher than the productivity of 0.66 g/l/h achieved in batch fermentation with free cells.

2.4 Materials for Cells Immobilization

2.4.1 Brick

Brick is readily available and inexpensive material. It is ease of use for immobilization. From previous study, there has no limitation of nutrient transfer in the batch on bricks with immobilized cells for ABE fermentation (Yen *et al.*, 2011). Immobilized cells fermentation on bricks can improve ABE fermentation in term of productivity (Qureshi *et al.*, 2000; Yen and Li, 2011).

The compositions of element in raw material for brick are silicon dioxide, aluminium dioxide and iron oxide subsequently. The silicon dioxide make

more brick's strength. For aluminium dioxide and iron, they change the brick pigment to red after sintering (Sutas *et al.*, 2012).

Table 2.4 Chemical composition of brick (Sutas *et al.*, 2012)

Substance	Proportion (%)
SiO_2	60.67
Al_2O_3	15.18
Fe_2O_3	7.61
K_2O	3.12
MgO	1.15
TiO_2	1.18
CaO	0.79
Na_2O	0.56
SO_2	0.55
MnO_2	0.22
BaO	0.11
ZnO	0.01
ZrO	0.01

2.4.2 Activated Carbon

Active carbon is a highly porous material (microporous) produced from a carbon-rich precursor by some form of chemical or physical activation. It is the most widely used of all the relatively cheap general-purpose adsorbents. Most commercial grades of activated carbon possess large internal areas located within disordered micropore structures. From previous study, activated carbon as solid carriers improve butanol productivity (Welsh *et al.*, 1987).

Activated carbons are manufactured from relatively heterogeneous base materials such as bituminous or sub-bituminous coal, lignite, peat, coconut shells, or wood. Activated carbons (including activated carbon fibers) can also be prepared from homogeneous polymeric base materials such as polyacrylonitrile, cellulose or phenolic resin. The manufacture of activated carbons involves two

important steps: (1) carbonization and (2) activation (Knappe, 2006). The carbon family having a planar sp2 bonding is represented by graphite, where the layers of carbon hexagons are stacked in parallel (Inagaki, 2013). Graphite is the stable form of elemental carbon at ambient temperature and pressure.

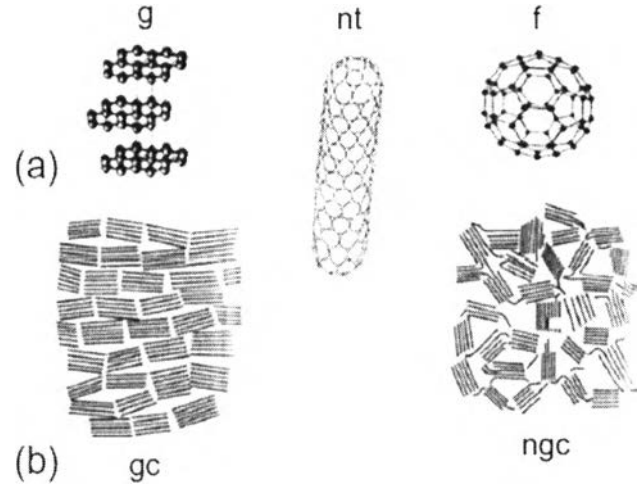


Figure 2.8 (a) Structures of graphite (g), carbon nanotube (nt) and fullerene (f). (b) Graphitizing carbon (gc) and non-graphitizing carbon (ngc) (Sing, 2014).

There are numerous investigations of the adsorptive properties of a wide variety of such carbons with Brunauer-Emmett-Teller (BET) areas between 5 m^2/g to 3000 m^2/g (Sing, 2014). A typical elemental composition of activated carbon is approximately 88% C, 6-7% O, 1% S, 0.5% N, and 0.5% H with the remainder being mineral matter (i.e., ash) (Knappe, 2006).

2.4.3 Zeolite 13X

Zeolite molecular sieve 13X is crystalline with highly porous aluminosilicate minerals and high surface area for bacterial cell adsorption. From previous study (Djukić-Vuković *et al.*, 2013), it is used to immobilized *Lactobacillus rhamnosus* for improve lactic acid production. Zeolite 13X has solve major disadvantage is the relative weakness of the adsorptive binding forces of bacteria and zeolite's buffering capacity can be controlled pH drop during fermentation.

Immobilized *Lactobacillus rhamnosus* onto zeolite has good system stability with high lactic acid concentration.

Zeolite 13X's composition is 1 Na_2O: 1 Al_2O_3 : 2.8 ± 0.2 SiO_2 : xH_2O and the sodium form represents the basic structure of the type X family, with an effective pore opening in the 910¼ range (Sigma-Aldrich, 2012). It has pore diameter 10 Å and surface area 730 m^2/g (Payne and Abdel-Fattah, 2005; Sigma-Aldrich, 2012). The chemical compositions of zeolite 13X were investigated and summarized in Table 2.5 (Zheng *et al.*, 2008).

Table 2.5 Chemical composition of zeolite 13X (Zheng *et al.*, 2008)

Substance	Proportion (%)
SiO_2	43.87
Al_2O_3	25.67
TiO_2	0.13
Fe_2O_3	1.8
FeO	0.21
MnO	0.45
MgO	1.04
CaO	2.05
Na_2O	9.56
K_2O	3.38
P_2O_5	0.22
H_2O	11.62

Therefore from all literature reviews, zeoilite, brick, and activated carbon were chosen for immobilized material to improve butanol production from ABE fermentation process.