CHAPTER II
LITERATURE REVIEW

2.1 Biobutanol

The butanol production is generally produced by Clostridia (e.g. *Clostridium beijerinckii* BA101, *Clostridium acetobutylicum* CGMCC5234, *Clostridium butyricum* TISTR1032, *Clostridium sporogenes* TISTR1452). Figure 2.1 shows a metabolic pathway for Acetone-Butanol-Ethanol (ABE) production in *Clostrium acetobutylicum*. *Clostridia* cultures are exactly anaerobic bacteria which use many carbon sources, such as monosaccharides and polysaccharides. From Figure 2.1, glucose is changed through the glycolytic (EMP) pathway producing pyruvate and generating 2 molecules of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). The initial phase is called acidogenic phase that generates acetic and butyric acids. So, the first phase occurs an active growth of the organic acids is important for generation of ATP needed for celled growth and metabolism. In this phase, butyrate is produced more than acetate which the organism can regenerate the NADH formed in glycolysis by its oxidation. Then, the metabolism will shift to the second phase of ABE fermentation, called solventgenic phase, when the acid concentrations reach a threshold value (around 60 mmol/l). The acids produced during the acidogenesis are used to produce acetone, butanol, and ethanol. The solventogenic phase is a small phase between acidogenic phase and sporulation that initiated by activation of transcriptional regulator Spo0A with pivotal role in sporulation, as shown in Figure 2.1.

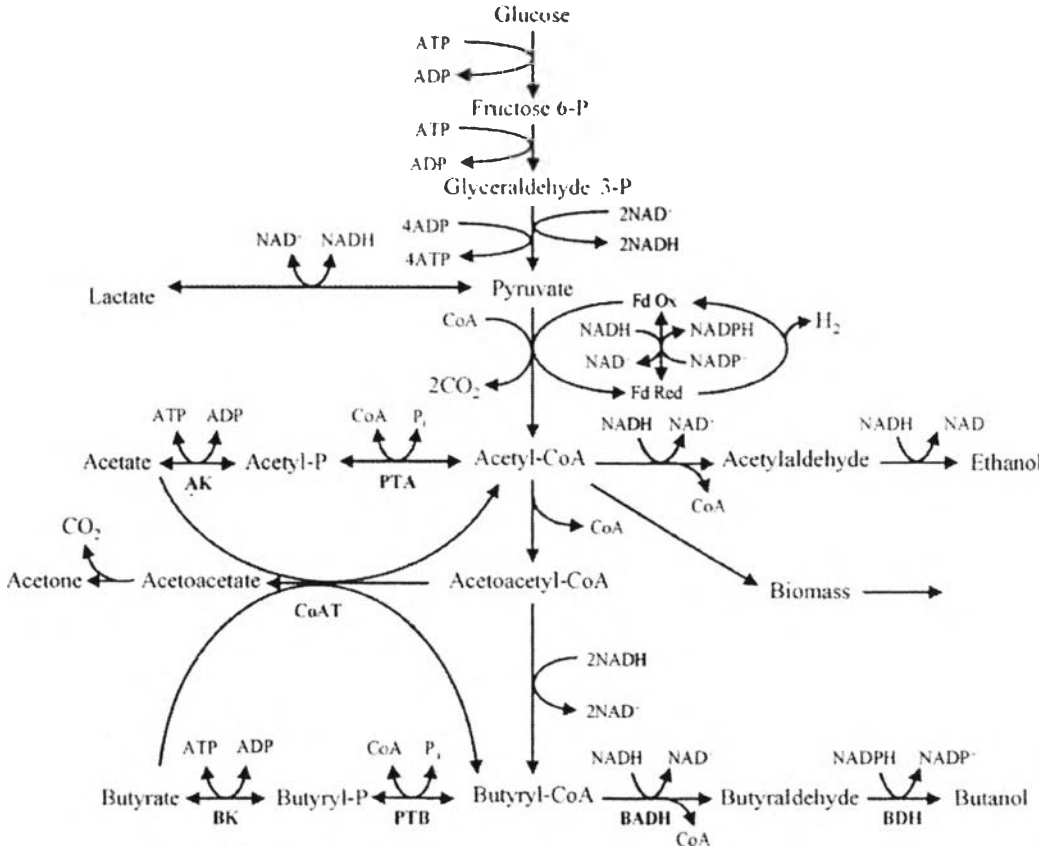


Figure 2.1 Metabolic pathways of *Clostrium acetobutylicum* ATCC 824 which enzymes are abbreviated as follows: PTA, phosphotransacetylase; AK, acetate kinase; CoAT, CoA transferase; PTB, phosphotransbutyrylase; BK, butyrate kinase; BADH, butyraldehyde dehydrogenase; BDH, butanol dehydrogenase (Shinto *et al.*, 2008).

The designing fermentations had been studied to maximize the butanol productivity. Batch fermentation is a simple model which reduces the risk of contamination. Fed-batch fermentation is more suitable for industry than batch reactor because this model gives higher concentration and productivity that overcomes the substrate and product inhibitors during fermentation. Continuous fermentation is an interesting model which has a shorter preparation time (lag phase) and higher productivity than another model because of physical property product and toxicity system. The physical property of ethanol is completely miscible with water while n-butanol is immiscible with water that separate into an ethanol-rich and n-

butanol-rich phase. However, the concentration of n-butanol in batch fermentation might less than one third of the n-butanol solubility in water. Thus, continuous model will produce the butanol more than batch model which does not have a product removal (Jang *et al.*, 2012)

Fermentation was mostly operated in a batch model for long times ago. The advantage of batch model is easier to operate and has lesser risks of contamination, but it gives lower productivities. In order to get a highly productive and cost competitive butanol fermentation system, the fermentation has been developing new strategies using inexpensive carbon substrates and designing new fermentation models. A conventional batch which this ABE fermentation yields solvents more than 12-20 g/l with fermentation time about 2 to 6 days. Because of solvent toxicity and limitation of sugars, the fermentation should be operated by diluted sugar solution with large process volume that makes this process uneconomical. So, continuous culture and fed-batch fermentations are the alternative methods that can remove and recovery fermentation broth (Lee *et al.*, 2008).

Fed-batch operation is an industrially preferred fermentation strategy as it allows higher concentration and productivity of desired product that can overcome the substrate and product inhibitors during ABE fermentation (Jang *et al.*, 2012). For example, Ezeji and co-workers (2007) applied an integrated fed-batch fermentation - gas stripping product recovery system with concentrated substrate feeding. This fermentation was operated for 201 h and a total of 500 g glucose was consumed to produce 151.1 g butanol with the yield and productivity of 0.30 g/g and 0.75 g/l/h, respectively.

Continuous fermentation is a good strategy that can reduce the preparation time lag phase (when compared to Batch and Fed-batch operation) and improve the productivity. However, the simple continuous model has a serious problem of cell wash-out at high dilution rate that affects the productivity. This problem can overcome by using high cell density cultivation which is particularly well suited when the cell grows slowly or it is strongly affected by product inhibition (reduced toxicity).

Table 2.1 Fermentation of *Clostridium sp.* (Sukumaran *et al.*, 2011)

Organism	Type of Fermentation	C-Source	Method for in Situ Product Removal	Solvent Conc. (g/L)	Yield (g/g)	Productivity (g/l/h)	Reference
C. saccharoperbutylacetonicum	Batch	Glucose	Liquid-Liquid Extraction	29.8	0.4	0.55	Ishizaki et al. (1999)
C. beijerinckii	Batch	Glucose	Pervaporation	51.5	0.42	0.69	Qureshi and Blaschek (1999)
C. acetobutylicum DSM 1731	Batch	Domestic Organic Waste	Nil	9.39	ND	ND	Claassen et al. (2000)
C. beijerinckii BA101	Continuous (immobilized on clay bricks)	Glucose	Nil	7.9	0.38	15.8	Qureshi et al. (2000)
C. beijerinckii BA101	Batch	Packing peanuts	Nil	27.7	0.37	0.2	Jesse et al. (2002)
	Batch	Agro waste	Nil	14.8			
C beijerinckii BA101	Batch	Spray dried molasses	Nil	10.7	ND	ND	Qureshi et al. (2001)
C. acetobutylicum ATCC824	Continuous	Low-grade glycerol-glucose mixtures	Nil	7.9	0.29	0.33	Andrade and Vasconcelos (2003)
C. beijerinckii	Batch	Packing peanuts	Nil	18.9	0.32	0.17	Ezeji et al. (2003a)
	Continuous	Packing peanuts		8.4	8.4	0.27	
C. beijerinckii BA101	Batch	Glucose	Gas stripping	75.9	0.47	0.61	Ezeji et al. (2003b)
C. beijerinckii BA101	Continuous	Glucose	Gas stripping	460	0.4	0.91	Ezeji et al (2004a)
C. beijerinckii BA101	Feb batch	Glucose	Gas stripping	233	0.47	1.16	Ezeji et al. (2004b)
C. acetobutylicum	Continuous (immobilized on fibrous matrix)	Glucose	Nil	5.1	0.42	0.46	Huang et al. (2003)
C. beijerinckii	Continuous (immobilized on membrane)	Corn Steep Liquor	Nil	6.2	0.3	2.01	Qureshi et al. (2004)
C. acetobutylicum P260	Batch	Corn fiber arabinoxylan	Gas stripping	24.67	0.44	0.47	Qureshi et al. (2006)
C. beijerinckii	Fed batch	Saccharified liquefied corn starch	Gas stripping	81.3	0.36	0.59	Ezeji et al. (2007c)
C. beijerinckii P260	Batch	Wheat straw hydrolysate	Nil	20.1	0.41	0.28	Qureshi et al. (2007)
C. beijerinckii ATCC 55025	Continuous (immobilized on corn stalk)	Glucose	Nil	5.1	0.32	5.06	Zhang et al. (2009)
C. beijerinckii	Batch	Wheat bran	Nil	11.8	0.32	0.16	Liu et al. (2010)
C. saccharoperbutylacetonicum N1-4	Batch	Cassava starch	Nil	21	0.41	0.44	Thang et al. (2010)
		Cassava strips	Nil	19.4	0.38	0.4	

ND, not determined/not mentioned in the study

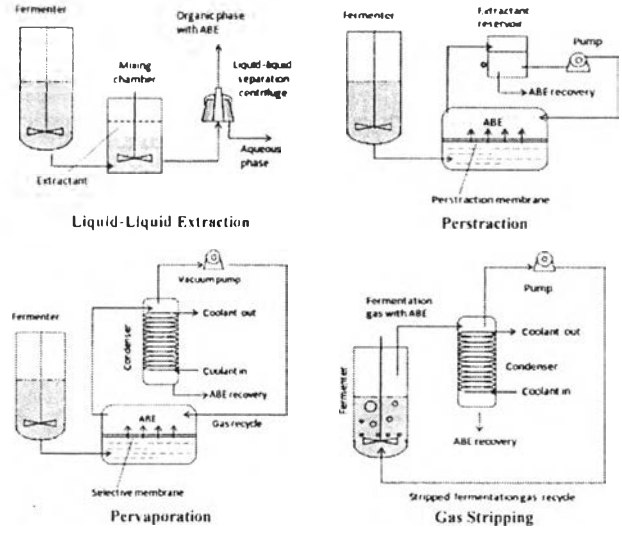


Figure 2.2 Production removal techniques (Sukumaran *et al.*, 2011).

The high cell density can be operated by immobilization or cell recycling. Cell immobilization is a physical localization of intact cells to a certain defined region of space with the maintenance of some desired activity. The support for the cell immobilization permits the exchange of substrates, products, inhibitors, etc., while separating the catalytic cell biomass from the bulk phase containing substrates and products. The ideal support should be inexpensive, stable, reusable, and nontoxic. This technique has two main types: carrier binding and entrapment. Carrier binding is a direct binding of cells to water-insoluble carriers by physical adsorption or ionic/covalent bonds. This adsorption of cells is the simplest and longest known mode of immobilization, because naturally cells spontaneously adsorb to the surface of insoluble material. Another immobilization is entrapment which based on the enclosure of cells within a rigid network. The entrapping itself is tight enough to prevent the release of the cells while it allows the diffusion of substrates and products.

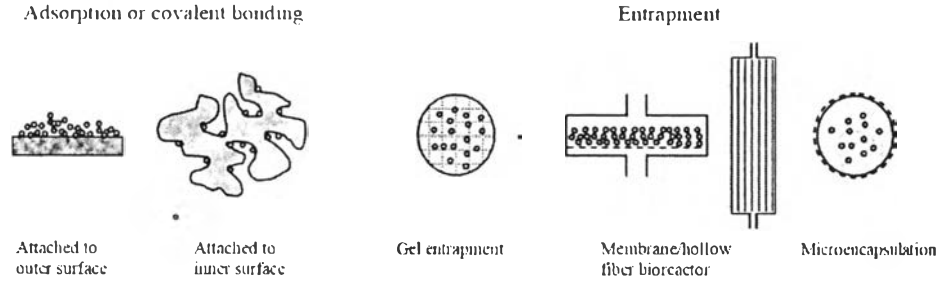


Figure 2.3 Adsorption and Entrapment of Cell immobilization (Zhu, 2007).

However, immobilized cells often suffer from a higher concentration of inhibitory products due to their higher productivity and mass transfer limitation. From previous work, Liu and co-workers (2009) described that butanol producing bacterial stain rarely tolerate more than 2% butanol. Butanol, as an organic solvent, tends to partition into the cytoplasmic membranes and changes the membrane structures. The microbial cell synthesis to increased levels of saturated levels of saturated acyl chains that accompanied by decreased unsaturated chains. Other works found an increase in longer acyl chain fatty acids at the expense of shorter acyl chains. Nielsen and co-workers (2005) studied the tolerance of *Staphylococcus haemmolyticus* which its membrane fatty acids responded the solvent concentration. The proportion of anteiso fatty acids increased from 25.8-33.7% while the proportion of 20:0 straight – chain fatty acids decreased from 19.3-10.1% that is similar to the *Clostridium*. Therefore, these will affect the cell membrane permeability and fluidity.

2.2 Treatment

Sekaran *et al.*, (2013) prepared the activated carbon from rice husk that is the solid waste in agricultural industry for using in wastewater treatment. Some organic compounds in wastewater can be treated by microorganisms which use an immobilization to protect them from shock load application and toxicity of chemicals in bulk liquid phase. Mesoporous activated carbon (MAC) was used as the carrier matrix for immobilization of *Bacillus* sp. for treatment sulphonated phenolic (SP) compounds in wastewater. Mesoporous activated carbon was prepared by pre-

carbonization and chemical activation with different heating temperatures, 700, 800, and 900 °C, which called MAC_{700}, MAC_{800}, and MAC_{900}, respectively. The fermentation which has a media 50 ml with *Bacillusi* sp. was operated by immobilization (1 g MAC) and free cell.

The nitrogen adsorption/desorption isotherms of precarbonized carbon (PCC) doesn't as good as MAC which is a type IV isotherm with a mesoporous matrix. The increase in uptake of nitrogen with increasing temperature is affected by the increasing of porosity created from carbon and silica components. The pore size of carbon treated with chemical impregnation and heat treatment temperature of 800 °C treatment has the largest average pore diameter. These pores was generated by the heat treatment which removes some unorganized carbons or residual tar materials.

Table 2.2 Average pore diameter, mesoporosity and production yield of mesoporous activated carbon (Sekaran *et al.*, 2013)

Sample	Average pore diameter Å	V_{meso}/V_{tot} (%)	Production yield of carbon %
C700	38.82	66.78	40.66
C800	39.36	68.41	39.19
C900	35.28	69.33	37.69

From X-ray results, the activated carbon prepared at high temperature formed on aromatic carbon with sp^2 bonding as graphitic structure that is more stable than an amorphous-like carbons with sp^3 bonding (MAC stable than PCC). The results of surface morphology confirmed the pore formation from treatment that should volatile some materials, e.g. ligins and some organic components (as shown in Figure 2.4). The sizes at mouth pore and interior pore formed macro pores and meso-micro pores, respectively.

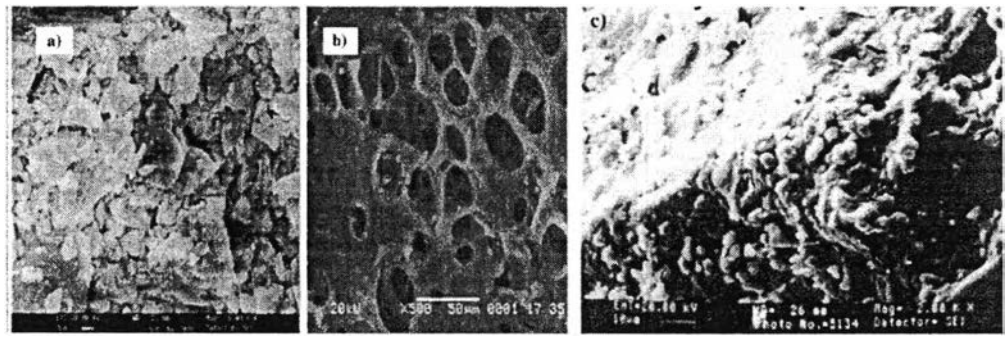


Figure 2.4 Scanning electron micrograph images (a) The precarbonized sample (MAC); (b) Activated carbon sample (MAC_{900}); (c) *Bacillus* sp. immobilized in the mesoporous activated carbon (Sekaran *et al.*, 2013).

The *Bacillus* sp. can attach into macro pores stronger than micro pores which microbial develops cohesion with the walls of the pores though extra cellular fibrils produced by the bacteria, so these microbial are hard to remove from the pores. Not only material but also pH can affect the fermentation which point of zero charge (PZC) of *Bacillus* sp. and MAC_{900} are 4.50 and 6.66, respectively. The initial increase of immobilization is caused from electrostatic attraction after that electrostatic repulsion has decreased the immobilization which electrostatic force occurs between exocellular membrane and MAC surface. Another studied factors are particles size, temperature, contact time, and mass of MAC_{900}, which affect the increase of internal/external surface area and the number of active sites for immobilization. Finally, the best conditions for wastewater treatment with *Bacillus* sp. are using pH 7.0, temperature 20 °C, and 1 g MAC_{900} with particle size 600 μm (Sekaran *et al.*, 2013).

Chen *et al.*, (2004) found that surface property of activated carbon can be modified by acid and basic that will change the surface chemistry, specific surface area, and porosity of the carbons. Treatment with activated carbon, which is Filtrasorb 200 carbon from Calgon Corp. (Pitts- burgh, PA), 20-23 mesh had been washed by deionized water (called DI-AC) and using HCl, HNO_3, and NaOH.

In the HCl modification, the DI-AC carbons were treated by a concentrated HCl solution (37% HCl), HA37-AC, at a ratio of 1 g/10 ml and shaken at 60 °C for 6

h. In the HNO_3 modification, the DI-AC carbons were respectively treated by 10, 30, %50, and concentrated HNO_3solutions (69 %HNO_3at a ratio of 1g carbon/ 10ml HNO_3 solution and shaken at60 °C for 6 h. The carbons were named NA -10AC, NA-30AC, NA-50AC, and NA-69AC. The treated carbons (DI-AC, HA37-AC, and NA69-AC) were treated by 1 M NaOH solution for 2 h. They were referred to as SH-AC, HA37-SH-AC, and NA69-SH-AC.

Table 2.3 List of carbon in the study (Chen *et al.*, 2004)

carbon type	brief description of modification
DI-AC	Filtrasorb 200 washed by DI water
HA37-AC	DI-AC treated by concentrated HCl and washed by DI water
NA10-AC	DI-AC treated by 10% HNO_3 at a ratio of 1 g/10 mL and washed by DI water
NA30-AC	DI-AC treated by 30% HNO_3 at a ratio of 1 g/10 mL and washed by DI water
NA50-AC	DI-AC treated by 50% HNO_3 at a ratio of 1 g/10 mL and washed by DI water
NA69-AC	DI-AC treated by concentrated HNO_3 at a ratio of 1 g/10 mL and washed by DI water
SH-AC	DI-AC treated by 1 M NaOH and washed by DI water
HA37-SH-AC	HA37-AC treated by 1 M NaOH and washed by DI water
NA69-SH-AC	NA69-AC treated by 1 M NaOH and washed by DI water

[a] AC, activated carbon; DI, deionized; HA, hydrochloric acid; NA, nitric acid; and SH, sodium hydroxide. The numerical value in the abbreviation indicates the volume percentage.

The results of specific surface area, carbon pH, and total acidity capacity (TAC) are shown in Table 2.4. It indicates that the acid-base treatment have no significant effect on the surface area. Because HCl and HNO_3 are inorganic acids and have small molecular weights while the treatment with organic acids such as citric acid, which has higher molecular weights, might decrease the surface area when adsorbed on activated carbons.

Table 2.4 Surface properties of studied carbon (Chen *et al.*, 2004)

Carbon type	Specific surface area (m2/g)	Carbon pH	TAC (mmol/g)
DI-AC	648	6.88	0.15
SH-AC	664	10.71	0.16
HA37-AC	659	5.71	0.19
HA37-SH-AC	662	8.49	0.19
NA69-AC	636	3.74	0.51
NA69-SH-AC	647	7.49	0.37

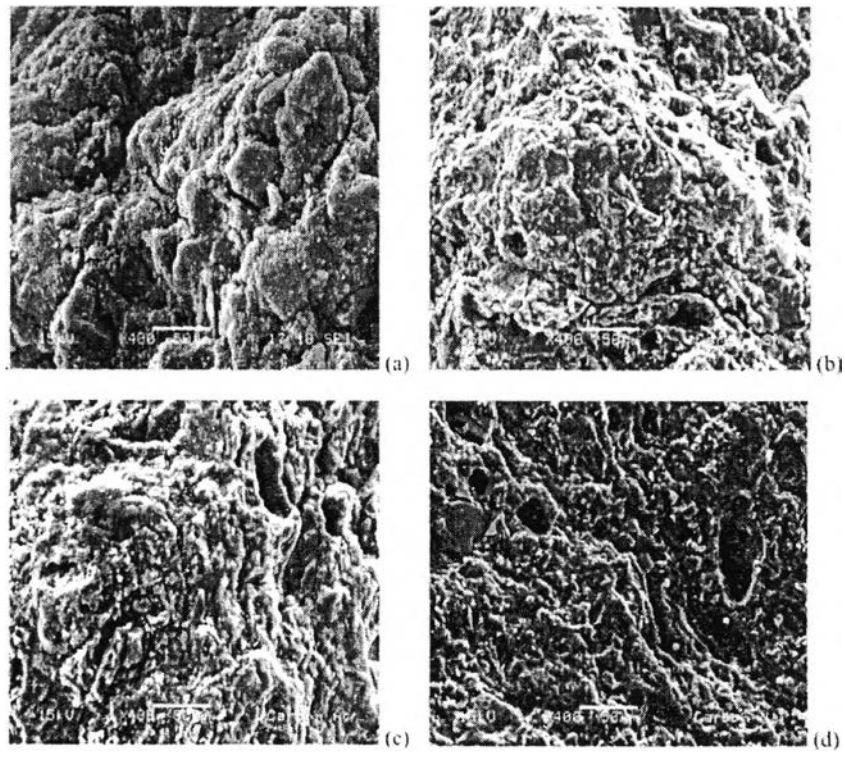
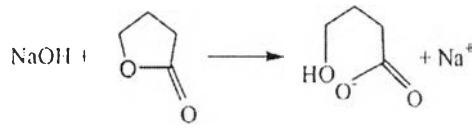


Figure 2.5 SEM images: (a) DI-AC; (b) SH-AC; (c) HA37-AC; and (d) NA69-AC (Chen *et al.*, 2004).

It was found that for the big molecular sizes, organic acids might block the pores on activated carbon. The acid treatments with HNO_3 and HCl have greatly reduced carbon pH values because stronger and weaker acidic functional groups are deprotonated at a lower pH and higher pH, respectively. HNO_3 treatment will increase the number of strong acid functional groups while HCl treatment will increase the number of weak acidic functional groups. Whereas, NaOH treatment has clearly increased the carbon pH values. TAC has been used to determine the quantity of oxygen functional groups. From Table 2.4, the NA69-AC sample has a TAC values more than DI-AC because the oxygen containing functional groups had been increased by HNO_3 treatment.

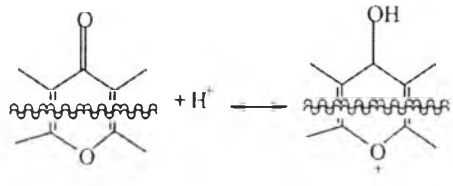
NaOH treatment might eliminate some acidic functional groups because the TAC value of NA69-SH-AC is higher than DI-AC but it is lower than NA69-AC.

The surface functional groups on the activated carbon can be determined by FT-IR analysis. NaOH treatment increases the hydroxyl groups such as lactone, as shown in Equation 2.1.



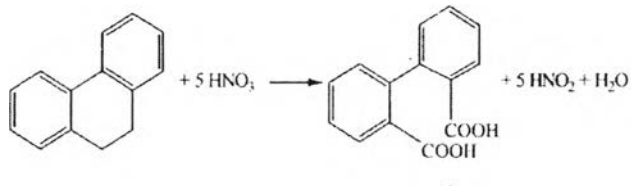
Eq 2.1

HCl treatment increases the single-bonded oxygen functional groups such as phenol, ether, and lactone, as shown in Equation 2.2.

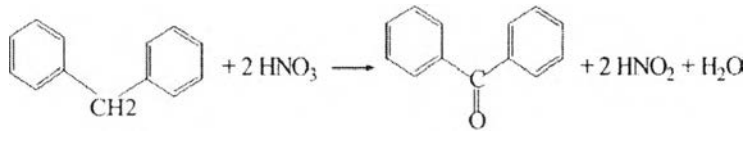


Eq 2.2

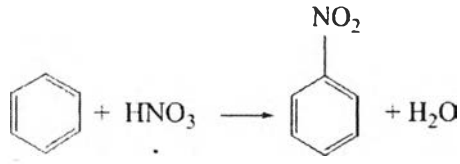
HNO_3 treatment generates a lot of carbonyl, carboxyl, and nitrate groups on surface which has the observable peaks at 1380-1400 and 3200-3600 cm^{-1}. The increasing of oxygen content from HCl treatment is caused by delocalized π electrons in the carbon basal plane that decrease the pH value of HA37-AC. Thus, HNO_3 treatment clearly decreases the percentage of surface graphitic and aromatic carbon and increases the percentage of oxygen and nitrogen which shows the mechanical in Equations 2.3, 2.4, and 2.5.



Eq 2.3



Eq 2.4



Eq 2.5

XPS was used to study the binding energy, as shown in Table 2.5. HCl and NaOH treatments have slightly changed the relative peak area percentage (RPA%) of aromatic and graphitic carbon, but the RPA% of single-bonded and double-bonded oxygen functional groups have increased and decreased, respectively. These results indicated the HCl and NaOH treatment might change carbonyl or carboxyl groups to become phenol or lactone groups following the mechanical Equations 2.1 and 2.2. Thus, HNO_3 treatment exhibited the decrease of RPA% aromatic-aliphatic and the increase of RPA% of all single-double bended oxygen functional groups and carboxylic groups following the mechanical Equations 2.3-2.5. All of these treatments have been applied with copper adsorption which HNO_3 treatment had the highest adsorption capacity because it enhance both oxygen and nitrogen compositions and hydroxyl and carboxylic groups.

Table 2.5 The relative peak area percentage of carbon (C 1s) peaks (Chen *et al.*, 2004)

Carbon type	BE of 284.5	BE of 286.0	BE of 287.2	BE of 288.5	BE of 290.0	BE of 291.5
DI-AC	67.13	11.69	6.22	5.38	5.10	4.48
SH-AC	66.73	15.11	5.98	4.88	4.21	3.09
HA37-AC	66.35	15.62	5.50	5.19	3.66	3.68
NA69-AC	57.54	16.16	7.98	9.99	4.48	3.85

2.3 Fermentation

Chen *et al.*, (2013) studied *Clostridium acetobutylicum* CGMCC5234 cells which immobilized onto pretreated cotton towels which treated with

polyethyleneimine solution, hydrochloric acid, and glutaraldehyde. Batch fermentation were performed from 60 g/l glucose, xylose, and the mixture of both sugars by both immobilized and free cells. As shown in Table 2.6, butanol concentration of 12.3 and 9.58 g/l were produced from glucose with immobilized and free cells, respectively. Xylose as medium can produce butanol concentration 10.02 and 8.42 g/l while mixed sugars produced butanol concentration of 11.1 and 8.78 g/l using immobilized and free cells, respectively. The immobilized cell fermentation had a higher total ABE than free cells, as illustrated in Table 2.6. These results showed that immobilized cells have stronger tolerance to butanol toxicity than free cells while butanol might be an inhibitor for cell growth and ABE production. Microbial could consume xylose and mixed sugars to generate ABE solvents, but less effectively than glucose that indicated xylose-grown cells have more inhibitory effect than glucose-grown cells. As a results, the immobilized cells consumed sugars faster and more efficiently than free cells which suggested that immobilization increased cellular activities and tolerance to butanol.

Table 2.6 ABE production from different sugar sources (60 g/l) with free and immobilized *Clostridium acetobutylicum* cells at 37 °C (Chen *et al.*, 2013)

Sugar sources	Operation	Utilized sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetoin (g/L)	Butanol yield[a] (g/g)	Total ABE[c] (g/L)
Glucose	I	59.8	5.879	12.3	1.629	1.35	0.205	19.81
	S	48.79	2.224	9.58	1.21	2.65	0.196	13.01
Xylose	I	50.1	4.112	10.02	0.20	0.11	0.200	14.33
	S	39.8	2.254	8.48	0.94	0.15	0.213	11.67
Sugar Mixture[b]	I	58.56	5.579	11.1	1.74	0.74	0.190	18.42
	S	46.8	2.577	8.65	1.85	1.08	0.185	13.08

[a]Butanol yield was based on the ratio of butanol concentration to utilized sugars.
[b]Sugar mixture: 30 g/L of glucose + 30 g/L of xylose.
[c]Total ABE includes acetone, butanol and ethanol except acetoin.
I: immobilized cells
S: suspended cells.

From Table 2.6, the free cells produced more acetoin than the immobilized cell, while the immobilized cells produced more butanol and acetone than the free cells. So, immobilized and free cells presented their distinctive selectivity on ABE production. Immobilized cells have a similar consumption sugar which glucose was quickly consumed and depleted at 45 h. When glucose concentration decreased

below 5 g/l, the consumption rate of xylose increased that could be due to the competition transport between glucose and xylose and glucose catabolic repression on key enzymes located in the xylose metabolic pathway. However, immobilized cells had a higher cell concentrations than free cells, but the free cells obtained a longer stationary phase than immobilized cells.

Butanol concentration increased with increasing initial glucose concentrations, butanol yields decreased and actually butanol concentration. Moreover, butanol concentrations had decreased with increasing high initial xylose concentrations. These results indicated that the cells had stronger tolerance to a high concentration of glucose than xylose. The high initial sugar concentrations might affect the inhibitor on butanol toxicity and sugar consumption. So, the initial sugar concentrations were important for butanol production which lower sugar concentrations produced higher butanol yields, productivities, and almost no residual sugars.

Fermentation was operated for the long-term fermentation of immobilized *Clostridium acetobutylicum* CGMCC5234 by repeated-batch fermentations using glucose and xylose. The average butanol concentration and productivity of glucose fermentation were 12.15 g/l and 0.325 g/l/h, respectively. Otherwise, the average butanol concentration and productivity of xylose fermentation were 9.09 g/l and 0.131 g/l/h that the conversion of xylose to butanol was less efficiently than glucose.

These experiments indicated the system had enhanced tolerance to butanol toxicity when microbial was immobilized on pretreated cotton towels than free cells. These microbial cells using glucose had a stronger tolerance to butanol than using xylose. The method of fermentation, immobilization and free cells, could affect the selectivity on ABE production too.

Medium is an important factor of fermentation. Welsh and co-workers (1987) studied mediums and solid carriers for *Clostridium acetobutylicum* ATCC824 immobilized materials are listed in Table 2.7. Medium A has only glucose, while medium B has glucose and some minerals. It was found that the immobilization gave a lower solvent production than free cell due to the nutrient adsorption by carriers. The medium in operation has been changed every three times for nine days that found little difference in start-up time and solvent yields throughout the

fermentation. The immobilized materials, which were coke, kaolinite or gel white, could be the suitable immobilized materials for continuous system. However, coke was selected to test because it was cheap and more readily available than kaolonite and gel white and it does not have pressure head build-up in the bioreactor. Whereas, another materials might occur because of their small aggregate size. The continuous fermentation was maintained for over 30 days without significant changes (Figure 2.6). The total solvent yield was 0.3 g total solvent/g glucose, the productivity was 1.12 g/l/h, and total solvent production was 12 g/l which presented the same results as free cell continuous system (Welsh *et al.*, 1987).

Table 2.7 Various solid immobilized by *Clostridium acetobutylicum* ATCC824 in batch culture on two different medium (Welsh *et al.*, 1987)

	MEDIUM A				MEDIUM B			
Support	Total[a] solvent (g/l)	Solvent[b] ratio	Butyric acid (g/l)	Yield[c]	Total solvent (g/l)	Solvent ratio	Butyric acid (g/l)	Yield
No solid added	12.8	4 : 1.4 : 1	1.3	.320	10.5	12.7 : 2.7 : 1	—	.263
T_{600} Silica	10.0	7 : 3.7 : 1	1.0	.250	7.5	6.7 : 2.1 : 1	3.49	.188
Kaolinite	10.0	9.3 : 3.0 : 1	0.8	.250	14.3	10.3 : 9.2 : 1	0.30	.358
Cab-o-sil	5.8	5 : 1.4 . 1	4.1	.145	4.7	3.7 : 1.3 : 1	3.71	.118
Vermiculite	12.0	16.1 : 7.6 : 2	1.9	.300	5.8	7.1 : 2.8 : 1	4.64	.145
Gel white	6.7	6.9 : 2.3 : 1	3.5	.168	9.8	9.4 : 2.3 : 1	0.62	.264
Activated carbon								
12 × 20 mesh	10.7	10.9 : 6.3 : 1	1.2	.268	7.7	8.8 : 3.3 : 1	3.00	.193
20 × 40 mesh	11.8	10.3 : 6.1 : 1	0.4	.295	6.7	6.8 : 2.9 : 1	3.76	.168
Coke	13.6	6.8 : 3.5 : 1	0.8	.340	8.0	8.5 : 3.1 : 1	3.96	.200

[a] Total solvent = butanol + acetone + ethanol
[b] Solvent ratio = butanol : acetone : ethanol
[c] Yield = g total solvent/g glucose added to the medium and based on complete use of glucose added to the medium

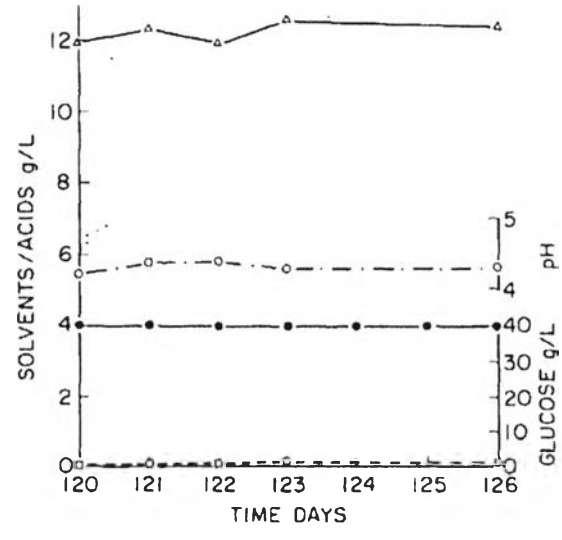


Figure 2.6 Change in glucose concentration, pH and solvent production with time during continuous culture with Clostridium acetobutylicum ATCC824 attached to coke as a support and using a semidefined medium at a dilution rate of 0.1 h^{-1}. △—△, total solvents; ○—·—○, pH; • —— •, influent glucose; □---□, effluent glucose (Welsh *et al.*, 1987).

Yen *et al.* (2011) studied the fermentation using *Clostridium acetobutylicum* BCRC10639 (the same as ATCC824) immobilized on brick, sponge, and nonwoven. Brick and nonwoven immobilizations exhibited higher ABE production than sponge immobilization which have a similar performance as free cells. Brick is more suitable than nonwoven fabric because of the higher density (easier to precipitate in the reactor) and easy availability (low price). The maximum butanol concentrations, after 60 h of fermentation, were 11.1 and 11.5 g/l in the batch with immobilized cells and free cells, respectively. Both immobilized cells and free cells had a similar butanol productivity of 0.109 g/l/h. Moreover, SEM results confirmed that the cells which were adsorbed onto the brick surfaces (Yen *et al.*, 2011).

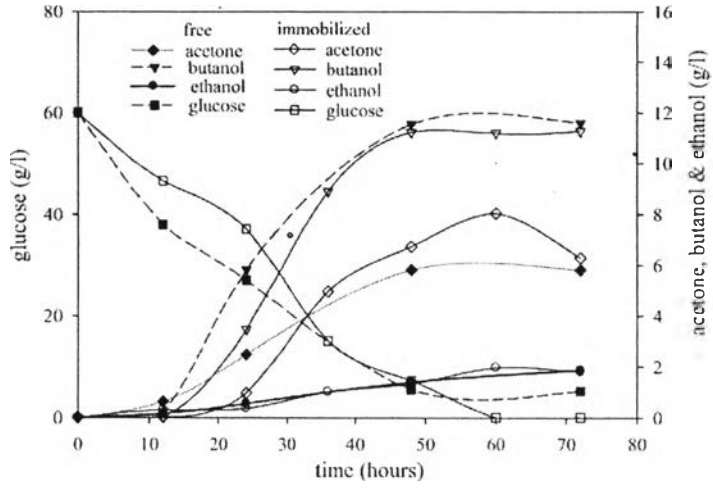


Figure 2.7 Glucose and ABE solvent results of free cells and immobilized cells (on bricks) with batch fermentation (Yen *et al.*, 2011).

From the results, they compared between unhydrolyzed sweet potato starch and glucose as carbon sources with immobilized batch fermentation on brick. The maximum butanol comcentration from glucose batch had a higher than the sweet potato starch batch which was 12.2 and 10.8 g/l, respectively, because these starch must be converted to sugars through the amylolytic step, subsequently to ABE production. The starch concentration rapidly decreased to almost zero within 12 h that solvent did not accumulate in this period. However, using starch as the carbon sources has a lower processing cost than using glucose.

The effect of particle size was studied to apply for immobilization with small (0.15-2.4 mm), medium (2.4-5.0 mm), and large size (more than 5.0 mm). A larger brick exhibited a butanol concentration less than a smaller size, whereas the smallest size (0.15-2.4 mm) gave similar butanol concentration as free cells. The batch with immobilized larger brick presented a higher levels of acetate and butyrate than both immobilized smaller brick batch and free cells batch. These results indicated that the metabolism pathway for ABE fermentation remained at acdiogenic phase, resulting in lower ABE production. Then, the brick was varied from 5 to 10, 15, and 20 g in fermentation medium. A greater amount of brick did not get a higher butanol concentration. The 20 g packing brick gave the lowest solvent productivity.

Another batch using 5-15 g bricks produced similar levels of butanol which the high packing density led to a limitation in the transport of nutrients.

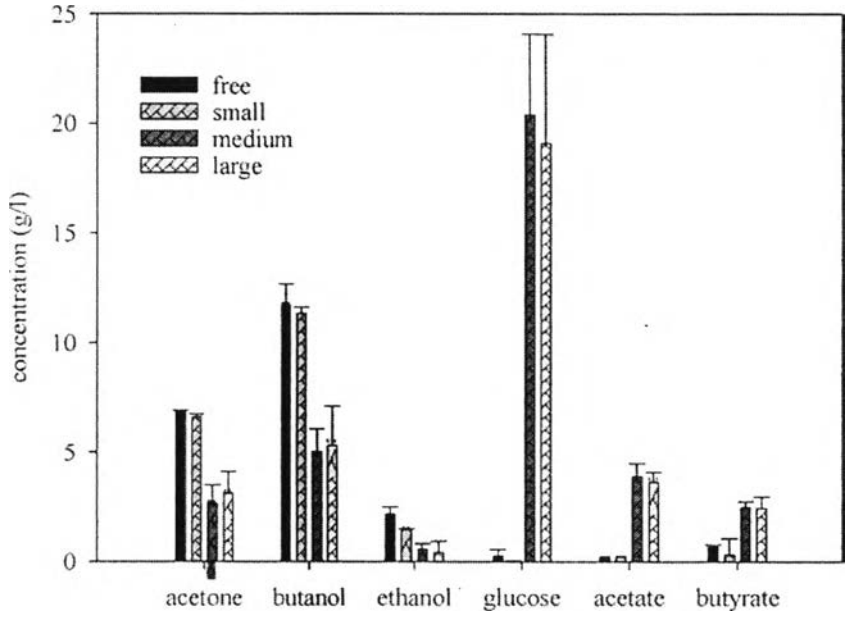


Figure 2.8 Effects of brick size to ABE fermentation at 60 g/l of glucose which used 5 g brick in 25 ml medium (Yen *et al.*, 2011).

Repeated batch results showed a stable solvent production from brick immobilized fermentation. The average butanol productivity from six repeated batch production cycles was 0.17 g/l/h, similar to the result of the free cell batch. These results indicated that microbial was adsorbed not only on the outside surface but also on the inside of the bricks.

The glucose solution 60 g/l with dilution rate of 0.054 h^{-1} and 0.108 h^{-1} was fed to the continuous fermentation with immobilized bricks. The pH level at 0.054 h^{-1} dilution rate was in the range of 4.0-4.3 that indicated the solventogenic stage. The average butanol productivity in both dilution rates was 0.48 and 0.71 g/l/h that was higher than the previous batch (0.19 g/l/h) and repeated batch (0.17 g/l/h). The total solvent concentration from continuous was 14.3±0.7 g/l/h and 11.1±0.6 g/l/h at dilution rate 0.054 and 0.108 h^{-1}, respectively.

The immobilized cell continuous culture system can improve reactor productivity and more stable reactors which clay brick was used in this work because of its low cost and availability. The fermentation used *Clostridium beijerinckii* BA101 with cooked meat medium, glucose, and P2 as the medium. Microbial cell was growing inside the reactor for 6 h, then P2 medium was fed with flow rate 6 ml/h. Continuous feed was allowed after 6 h of cell growth. The reactor was fed the P2 medium at low dilution rate with 0.3 h^{-1} for three days before samples were taken. Biomass accumulation and steady state was reached in 3-5 days (Qureshi *et al.*, 2000).

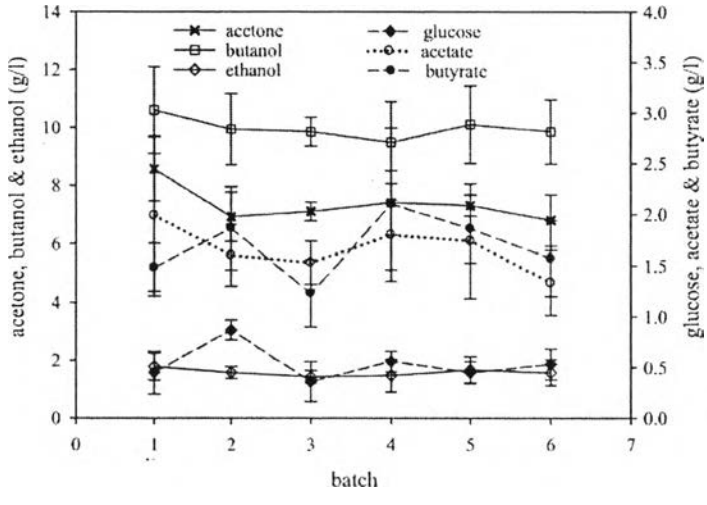


Figure 2.9 Results of repeated batch with brick immobilization (containing 60 g/l of initial glucose and 60 h of cultivation of each batch) (Yen *et al.*, 2011).

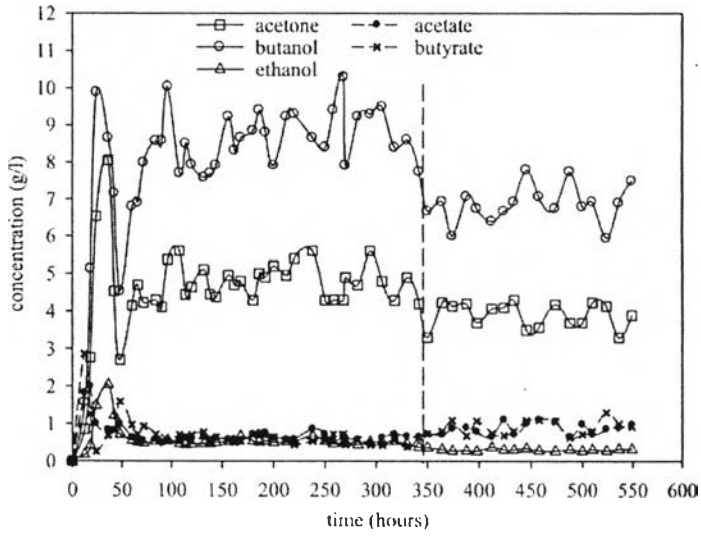


Figure 2.10 Solvents and acids in the effluent of continuous fermentation at the dilution rate of 0.054 h^{-1} and 0.108 h^{-1}. The dash line indicated the shift of dilution rate from 0.054 h^{-1} to 0.108 h^{-1} (Yen *et al.*, 2011)

As a result, the increase of solvent productivity occurred the dilution rate from 0.3 to 2.0 h^{-1} while a further increase from 2.0 to 2.5 h^{-1} gave a decreasing productivity. The dilution rate at 2 h^{-1} gave the highest productivity of 15.8 g/l h, total solvent concentration (ABE) 7.9 g/l, and glucose utilization 33% of feed. The steady operation with dilution rate 0.3 h^{-1} and 2.0 h^{-1} gave the total solvent 5.7 to 8.0 g/l and 6.2 to 10.3 g/l, respectively. From these results, all of dilution rates influenced the production fluctuated and increased with residence time. The continuous reactor was operated over a period of nearly 25 days that reactor stopped due to excessive microbial growth. Figure 2.11 shows SEM images that at the end of 25 days, the total cell concentration was 73.7 g/l (dry weight).

Productivity was at maximum at the dilution rate 2.0 $h^{-1}$and processed to decrease at higher this dilution rate. The decrease at a higher dilution rate was most likely due to active growth of microbial. This occurred the larger concentration of acids at high dilution rate during the active growth phase, and similar to the acid phase of the batch culture. The decrease of productivity at dilution rate 2.5 h^{-1} when compared to 2.0 h^{-1} was due to insufficient contact time between cells and feed medium that affected the acid productions at this dilution rate. The increase in cell

growth and acid production was also related a decrease in solvent yield which solvent yield was at maximum at the lowest dilution rate (0.3 h^{-1}) and decreased a little bit until the dilution rate was increased above 2.0 h^{-1} at which point increased amount of glucose was being consumed for growth and acid production. The decrease in solvent yield is considered with increased glucose utilization required for cell growth and acid production at higher dilution rates. So, the fermentation is possible to operate the reactor longer than 25 days if the dilution rate does not increase to 2.5 h^{-1} which a lot of cellular growth might block the reactor at high dilution rate. The reactor can be operated by limiting nutrients in the feed once enough biomass growth has occurred inside the reactor.

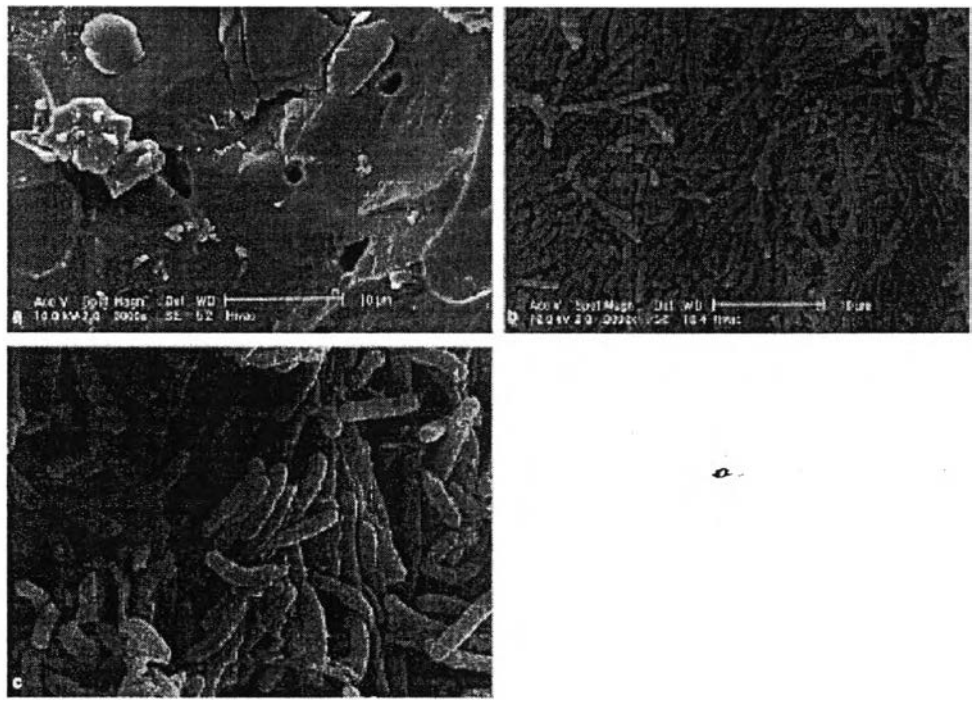


Figure 2.11 Scanning Electron Micrographs of immobilized cells of *C. beijerinckii* BA101 (a) Clay brick 3000x, (b) Immobilized cells 3000x, (c) Immobilized cells 5669x (Qureshi *et al.*, 2000).

The small oscillation in total solvent concentration might due to the high butanol production occurring at a rate faster than it can be removed from the area

surrounding the cell. The production was decreasing due to the effect of butanol toxicity.

The advantage of immobilized cell system is able to maintain high cell concentration, improve reaction rates, and stable at high dilution rates with little cell washout and simplicity of operation.

From these literatures, the activated carbon, which treated with suitable condition to adjust pH for microbial cells, might enhance the performance of cell immobilization. The immobilized cell will tolerance more than free cell and maintain for long-term butanol production, yield and productivity. The microbial cells might adsorb on the treated activated carbon and aggregate to form biofilms for protection from cruel environment. This work will study a treated activated carbon as a material for immobilization cell system to find the suitable treatment and the effect of treatment for ABE fermentation using *Clostridium beijerinckii* TISTR1461.