CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemical

1. Zeolite molecular sieve 13X, beads, 8-12 mesh (Sigma Aldrich)
2. Activated carbon, DARCO®, granular, 4-12 mesh (Sigma Aldrich)
3. Brick obtained from construction site
4. Sulfuric acid (H_2SO_4)
5. Hydrochloric acid (HCl)
6. D-Glucose anhydrous
7. Yeast extract
8. DifcoTM Cooked meat medium (CMM)
9. Potassiym dihydrogen (KH_2PO_4)
10. Dipotassium hydrogen (K_2HPO_4)
11. Amonium acetate (CH_3COONH_4)
12. Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)
13. Manganese (II) sulphate ($MnSO_4 \cdot H_2O$)
14. Iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$)
15. Sodium Chloride (NaCl)
16. Para-amino-benzoic acid
17. Thiamin
18. Biotin
19. Standard acetone, butanol, ethanol, acetic acid, and butyric acid
20. Propanol
21. *Clostridium beijerinckii* TISTR1461

3.2 Equipment

1. Autoclave
2. High Performance Liquid Chromatography (HPLC) with a refractive index detector using an Aminex-HPX 87H column (300 mm x78 mm,

Bio-Rad Lab, USA)

3. Gas Chromatograph equipped with a flame-ionization detector (GC-FID) using Innowax column.
4. Scanning Electron Microscope (SEM)
5. Brunauer-Emmett-Tellet (BET) Surface Area Analysis
6. X-ray Diffraction (XRD)
7. X-ray fluorescence (XRF)
8. UV-VIS 1800
9. Glassware
10. Oven
11. pH meter
12. Incubator shaker
13. Filter paper
14. Water bath
15. Centrifugal machine

3.3 Methodology

3.3.1 Preparation of Carriers for Cell Immobilization

For zeolite molecular sieves (type 13X, beads, 8–12 mesh, 1 Na_2O: $1Al_2O_3$: 2.8 ± 0.2 SiO_2: xH_2O) were purchased from Sigma Aldrich. Before utilization, 2 g of zeolite were treated with a 0.5% sulfuric solution, under agitation for 4 hours, then washed with and kept in distilled water for 24 hours and dried at 150 °C (Santos *et al.*, 2005).

For brick and activated carbon (sieving for particle size of 0.15–2.4 mm), 2 g of materials were washed with deionized water several times followed by sterilization in an oven at 250 °C for 2 hours. (Qureshi *et al.*, 2000)

3.3.2 Medium Preparation

For DifcoTM Cooked meat medium (CMM) (Qureshi *et al.*, 2008), 0.875 g of CMM pellet and 0.14 g glucose were dissolved with 7 ml of distilled water followed by sterilizing at 121 °C for 15 minutes and being cooled to room temperature subsequently. After that, one loop of cell spores was put into the prepared

solution and heat shock at 80 °C for 2 minutes to activate and diminish weak cultures. The CMM culture solution was kept in 37 °C without agitation and waited for cells activation within 24-30 hours.

3.3.3 Inoculum Development

To prepare for P2 medium inoculum (Qureshi and Blaschek, 1999), 1.8 g of glucose and 0.06 g of yeast extract were dissolved in 52.78 ml of distilled water followed by sterilizing at 121 °C for 15 minutes and being cooled to room temperature subsequently. A 7 ml of active growing cells (from liquid CMM) was inoculated into prepared P2 medium solution following by adding 500 µl of buffer (KH_2PO_4 50 g/l, K_2HPO_4 50 g/l, CH_3COONH_4 220 g/l), 100 µl of mineral ($MgSO_4 \cdot 7H_2O$ 20 g/l, $MnSO_4 \cdot H_2O$ 1 g/l, $FeSO_4 \cdot 7H_2O$ 1 g/l, NaCl 1 g/l) and 20 µl of vitamins (para-amino-benzoic acid 0.1 g/l, thiamin 0.1 g/l, biotin 0.001 g/l) to solution. After keeping P2 medium solution in 37 °C without agitation for 8 hours, cells were ready for fermentation step.

3.3.4 Fermentation

The ABE fermentation was conducted in a 250 ml, Duran, screw-capped bottle. The 100 ml of P2 medium contained glucose 60 g/l, acetate buffer, minerals, vitamins, and yeast extract. For immobilization batch the production medium was added by 2% (w/v) of porous material before was sterilized by an autoclave at 121 °C for 15 minutes and being cooled to room temperature subsequently. After that the 5 ml of prepared inoculums were transferred to the production medium. Before incubation, 99.99 % nitrogen gas was purged through the medium for 5 minutes at constant rate to assure anaerobic condition. Then the incubation temperature was maintained at 37 °C with a 150 rpm orbital shaking rate in an incubator shaker. Samples were collected after centrifuge and filtered fermentation products at 0, 12, 24, 48, 72, 96, and 120 hours to analyze pH, product concentration and glucose remain.

3.3.5 Repeated Batch Fermentation

Repeated batch fermentation was applied to immobilized cells without repeated inoculation. The supernatant was removed at the end of each cycle with keeping the cell immobilization on zeolite inside the fermentation bottle. The old medium was replaced by 100 ml of fresh fermentation medium. Each repeated batch

received 48 hours of cultivation in anaerobic conditions and collect the samples to analyze at begin and end of each cycle (Chen *et al.*, 2013).

3.4 Analysis Method

3.4.1 High Performance Liquid Chromatography (HPLC)

The quantity of glucose, acetate, butyrate, acetone, and butanol were determined by High Performance Liquid Chromatography (HPLC) equipped with a refractive index detector (Series 200 LC/S/N291N5060508, Perkin Elmer) using an Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA) and a guard column (30 mm x 4.6 mm, Bio-Rad Lab, USA) under these following conditions: flow rate 0.60 ml/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C, and 20 µl for injection volume.

3.4.2 Gas Chromatography (GC)

The acetone-butanol-ethanol concentration produced from ABE fermentation step was measured by a gas chromatograph (Series Perichrom 2100) equipped with a flame ionization detector using Innowax column length 30 m under these following conditions: N_2 flow rate 45 ml/minute, detector temperature 240°C, injection Temperature 240°C, and volume injection 0.5 µl.

3.4.3 UV-VIS Technique (UV)

The growth and density of cells was determined spectrophotometrically as the optical density at 600 nm (OD600nm) by UV-VIS Spectrometer (Shimadzu / UV 1800). Samples were diluted for appropriate dilution in deionized water before spectrophotometrically as the optical density at 600 nm and set deionized water as blank.

3.4.4 Scanning Electron Microscope (SEM)

The surface morphology and porosity of materials were observed by Scanning Electron Microscope (SEM) (JSM 6406 SEM instrument operated at 15 kV accelerated voltage). Samples were sputter coated with gold before analysis. There also using EDS function to identified the elements on materials surface.

For scanning electron microscopy (SEM) with cell immobilization material, samples were fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH

7.4) for 2 hours. This was followed by a thorough cleansing of the materials surface with 0.1 M phosphate buffer in order to remove all cells precipitated on the surface twice and clean with water. Samples were then dehydrated by graded ethanol series (30%, 50%, 70%, 95%, and 100%) and dry with critical point dryer). Samples were fixed to stubs using conductive silver paint, and then sputter-coated with gold. Microscopic observations were accomplished using a JEOL JSM-5410LV field emission scanning electron microscope at 15kV.

3.4.5 X-Ray Diffraction (XRD)

Materials crystallinity and element compound were determined by X-ray diffraction (Rigaku Model DMAX 2200 HV). Samples of each size fraction were analyzed. All samples were scanned from $2\theta = 5°$ to 80° with a step size of 0.01°. Determination time was 0.5 s/0.01°. In addition, the chemical structure of materials changing between untreated and treated.

3.4.6 Surface Area Analysis (BET)

BET surface area information of the materials were measured by N_2 adsorption/desorption measurements (Quantachrome instrument; model: BELSORP–max, BEL, Japan) done at 100 °C (373 K). The 0.5 g of sample was put into tube of the Quantachrome instrument and degassed using a vacuum for 24 hours. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves using BELSORP–max software.

3.4.7 pH Meter

Initial pH of materials was measure by pH meter (pH 700 Eutech Instrument) The materials were grinded by volume of water or adding drops of water to surface of materials to enable pH measurement with pH electrode. The ratio of weight materials per water was 1:9 (Bell and Labuza, 1992).

3.4.8 X-ray Fluorescence Spectroscopy (XRF)

The quantitative and qualitative elemental analysis of the materials were analyzed by XRF technique, Philips model PW 2400. With a primary X-ray excitation source from an X-ray tube, the X-ray can be absorbed by the atom, and transfer all of its energy to an innermost electron. During the process, if the primary X-ray has sufficient energy, electrons are ejected from the inner shell, creating vacancies. These vacancies present an unstable condition for the atom. As the atom re-

turns to its stable condition, electrons from the outer shells are transferred to the inner shells, and the process gives off a characteristic X-ray, whose energy in the difference between the two binding energies of the corresponding shells. Because each element has a unique set of energy levels, each element produces X-rays at a unique set of energies, allowing one to non-destructively measure the elemental composition of a sample. The intensities of observed lines for a given atom vary according to the amount of that atom present in the specimen.