CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

- Activated Carbon, DARCO®, Granular, 4-12 Mesh (Sigma Aldrich)
- Nitric Acid (HNO_3)
- Hydrochloric Acid (HCl)
- D-Glucose Anhydrous
- Yeast Extract
- Difco^{TM} Cooked Meat Medium (CMM)
- Potassiym Dihydrogen (KH_2PO_4)
- Dipotassium Hydrogen (K_2HPO_4)
- Amonium Acetate (CH_3COONH_4)
- Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$)
- Manganese (II) Sulphate ($MnSO_4 \cdot H_2O$)
- Iron (II) Sulphate Heptahydrate ($FeSO_4 \cdot 7H_2O$)
- Para-amino-benzoic Acid
- Thiamin
- Biotin
- Standard Acetone, Butanol, Ethanol, Acetic Acid, and Butyric Acid
- *Clostridium beijerinckii* TISTR1461

3.2 Equipments

- Autoclave
- High Performance Liquid Chromatography (HPLC) equipped with a refractive index detector using an Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA)
- Scanning Electron Microscope (SEM) using with EDX function
- Brunauer-Emmett-Tellet (BET) Surface Area Analysis

- Fourier Transform Infrared Spectroscopy (FTIR)
- UV-VIS Spectrometer 1800
- Glassware
- Oven
- pH Meter
- Incubator Shaker
- Filter Paper
- Centrifugal Machine

3.3 Experimental Procedures

3.3.1 Preparation of Immobilized Material

The activated carbon was treated by acid-base and amine-base treatment. Acid-Base treatment, the DARCO® activated carbon having particle size of 1.70-2.36 mm was washed with deionized water several times, denoted as DI-AC. The DI-AC was treated with 10%w/v nitric acid and shaken at 60°C for 6 h, denoted as NA-AC. These DI-AC and NA-AC were treated by 10%w/v 1M sodium hydroxide for 2 h, denoted as SH-AC and NASH-AC, respectively. Moreover, the DI-AC was treated by reflux with 3-aminopropyltriethoxysilane (APTES) and sodium hydroxide at 78 °C for 6 h, called AS-AC(R) and SH-AC(R) (Wang *et al.*, 2007). All AC samples were washed with deionized water after treatment until no variation in the pH of washed liquid was detected (Chen *et al.*, 2004).

3.3.2 Medium Preparation

For Difco™ Cooked meat medium (CMM), 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 min and being cooled to room temperature subsequently. After that, one loop of cell spores was put into the prepared solution and were heat shocked the suspension at 80 °C for 2 min to activate and diminish weak cultures. The CMM culture solution was incubated in 37 °C without agitation and waited for cells activation within 24-30 h (Qureshi *et al.*, 2008).

3.3.3 Inoculum Development

To prepare P2 medium inoculum, 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 min and being cooled to room temperature subsequently. A seven milliliters 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 h, cells were ready for fermentation step (Qureshi *et al.*, 1999).

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 min 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 min 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 h to analyze pH, product concentration and glucose remain.

3.4 Analytical Methodology

3.4.1 High Performance Liquid Chromatography (HPLC)

The quantity of glucose, acetate, butyrate, acetone, and butanol were determined by high performance liquid chromatograph (HPLC) equipped with a

refractive index detector (Series 200 LC/S/N291N5060508, Perkin Elmer) using an Aminex-HPX 87H column (300 mm x 78 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 50 µl for injection volume.

3.4.2 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 deionized water was used as blank.

3.4.3 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 EDX function to identify 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 h. 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.

3.4.4 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 at 250 °C for 24 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves using BELSORP–max software.

3.4.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used to determine the vibration frequency changes in the functional groups in the carbons. The spectra of carbons were collected by a Thermo Nicolet, NEXUS 670 within the range of 400-4000 cm^{-1}. Specimens of various activated carbons were first mixed with KBr and then ground in a mortar at an approximate ratio of 1/100 for the preparation of pellets. The resulting mixture was pressed at 10 tons for 5 min. Sixteen scans and 8-cm^{-1} resolutions were applied in recording the spectra.