CHAPTER III

MATERIALS AND METHOD

3.1 Materials

The detail of chemicals, apparatus and instruments is tabulated in Table 3 and 4.

Table 3: List of chemicals consumed during experimentation

Chemicals	Manufacturers
Nutrient agar	Oxoid, England
Brain heart infusion (BHI) broth	Oxoid, England
D-+-Glucose anhydrous	Bio Basic Inc., Canada
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
Ammonium nitrate (NH_4NO_3)	Acros Organics, UK
Magnesium sulfate ($MgSO_4$)	Acros Organics, UK
Ferrous sulfate ($FeSO_4$)	Fisher Scientific, UK
Calcium Chloride ($CaCl_2$)	Merck, Germany
Manganese sulfate ($MnSO_4$)	Merck, Germany
Ethylenediamine tetraacetic acid (EDTA)	Merck, Germany

Hydrochloric acid (HCl)	RCI Labscan, Thailand
Methanol	RCI Labscan, Thailand
Acetonitrile	RCI Labscan, Thailand
Trifluoroacetic acid (TFA)	Fischer Scientific, UK
Surfactin standard	Sigma Aldrich,St. Louis, USA

Table 4: List of apparatus and instruments consumed during experimentation

Apparatus/Instruments	**Manufacturers**
Petri dish	Friendemann Schmidt, Australia
Erlenmeyer flask with cap	Schott, Germany
Autoclave	Hirayama Manufacturing, Japan
Incubator	Memmert, Germany
Rotary shaker incubator	Sastec, Malaysia
Centrifuge	Hanil Science Industrial, S. Korea
pH meter	Mettler Toledo, Germany
High pressure liquid chromatography (HPLC) - Column: Zorbax 5µm C18 column - Detector: visible wavelength (VW)	Agilent Technologies, USA and Merck, Germnay (column)
Vacuum oven	Binder, Germany
Matrix-assisted laser desorption/ionization – time of flight (MALDI-ToF)	Bruker, USA

3.2 Methods

The procedures for both OFAT and RSM optimization are explained as below.

3.2.1 Culture maintenance and seed culture preparation

The organism used in this study is a local isolate *Bacillus subtilis* 3M which was obtained from the Microbiology Lab, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The latter was cultured on nutrient agar at 37 °C for 24 h and maintained at 4 °C. For the seed cultures, *B. subtilis* 3M from an agar slant was inoculated into 50 mL of sterile brain heart infusion (BHI) broth medium containing (g/L): brain infusion solids (12.5), beef heart infusion solids (5.0), proteose peptone (10.0), glucose (2.0), sodium chloride (5.0), disodium phosphate (2.5) and incubated in 100 mL conical flasks at 30 °C and 200 rpm for 24 h on a rotary shaker.

3.2.2 Optimization of media composition

For this optimization experiment, the parameters involved were glucose, ammonium nitrate, ferrous sulfate and manganese sulfate.

3.2.2.1 Inoculum preparation

For surfactin production, 5 mL of freshly grown culture from BHI broth culture was inoculated into 250 mL Erlenmeyer flask containing 100 mL of sterile inoculation of Cooper's medium as described by Cooper et al. (1981) which composed of glucose = 40 g/L, Na_2HPO_4 = 40 mM, KH_2PO_4 = 30 mM, NH_4NO_3 = 0.05 M, $MgSO_4$ = 0.8 mM, $CaCl_2$ = 7 µM, EDTA = 4 µM, $FeSO_4$ = 4 µM and $MnSO_4$ = 1.5 mM and incubated on a rotary shaker at 200 rpm for 24 h at 30 °C.

3.2.2.2 One-factor-at-a-time (OFAT) optimization study

One-factor-at-a-time (OFAT) optimization was used in order to determine the central value for future work on response surface methodology (RSM). The experiment was performed by changing only one variable at a time while other variables were kept at

constant value. The parameters subjected to OFAT optimization were glucose, ammonium nitrate, ferrous sulfate and manganese sulfate concentrations. Shake flask fermentation was carried out by using Cooper's media as well, though certain concentration of media components selected was varied according to the experimental design. In all cases, a 250 mL Erlenmeyer flask containing 100 mL of media was inoculated with 5% (v/v) inoculums and incubated at 30 °C in a rotary shaker at 200 rpm for 96 h. All experiments were carried out in triplicate.

3.2.2.3 Response surface methodology (RSM) optimization study

For this study, shake flask fermentation also was performed by using Cooper's media as the fermentation media. Concentration of selected media components was varied according to the experimental design obtained from the previous OFAT optimization study. In all cases, a 250 mL Erlenmeyer flask containing 100 mL of media was inoculated with 5% (v/v) inoculums and incubated at 30 °C in a rotary shaker at 200 rpm for 96 h. All experiments were carried out in triplicate.

Response surface methodology (RSM) was employed to study the interaction among four variables, which are glucose, ammonium nitrate, ferrous sulfate and manganese sulfate concentrations and their contribution towards surfactin and biomass production. A

factorial central composite design (CCD) for four factors with replicates at the centre point and star points was employed, which required 30 experiments. Table 5 represents the range and center point values of four independent variables.

Table 5: Ranges of variation for four independent variables used in RSM

Factors	Codes	Levels				
		-α	-1	0	+1	+α
Glucose (g/L)	A	20	30	40	50	60
Ammonium nitrate (M)	B	0.03	0.04	0.05	0.06	0.07
Ferrous sulfate (μM)	C	40	80	120	160	200
Manganese sulfate (mM)	D	0.5	1.0	1.5	2.0	2.5

3.2.3 Optimization of fermentation condition

For this optimization experiment, the parameters involved were fermentation time, inoculum volume and temperature.

3.2.3.1 Inoculum preparation

For surfactin production, 5 mL of freshly grown culture from BHI broth culture was inoculated into 250 mL Erlenmeyer flask containing 100 mL of sterile inoculation of modified Cooper's medium from media composition study by RSM which composed of glucose = 42 g/L, Na_2HPO_4 = 40 mM, KH_2PO_4 = 30 mM, NH_4NO_3 = 0.05 M, $MgSO_4$ = 0.8 mM, $CaCl_2$ = 7 µM, EDTA = 4 µM, $FeSO_4$ = 131 µM, $MnSO_4$ = 1.64 mM and incubated on a rotary shaker at 200 rpm for 24 h at 30 °C.

3.2.3.2 One-factor-at-a-time (OFAT) optimization study

The optimization process for fermentation condition was carried out by one-factor-at-a-time (OFAT) method where only a single factor was varied while the remaining factor constant was kept at constant. The optimal level of surfactin and biomass production was

studied by varying the fermentation time (24 h, 48 h, 72 h, 96 h, 120 h and 144 h), inoculum volume (1 %, 5 %, 10 %, 15 % and 20 %) and temperature (20 °C, 30 °C, 40 °C and 50 °C).

In all cases, freshly grown of inoculum broth culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml of optimized Cooper's media from media composition optimization study. Fermentation process was conducted by shake flask fermentation technique in a rotary shaker with 200 rpm. All experiments were carried out in triplicate.

3.2.3.3 Response surface methodology (RSM) optimization study

RSM coupled with a factorial central composite design (CCD) for three factors with replicates at the centre point and star points was used for optimization study. Table 6 represents the range and center point values of three independent variables which based on the preliminary OFAT experiments. In all cases, freshly grown of inoculum broth culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml of optimized Cooper's media from media composition optimization study. Fermentation process was conducted by shake flask fermentation in a rotary shaker with 200 rpm. All experiments were carried out in triplicate.

Table 6: Ranges of the three independent variables variation used in RSM

Factors	Codes	Levels				
		-α	-1	0	+1	+α
Fermentation time (h)	A	31.6	48	72	96	112.4
Inoculum volume (%)	B	3.3	5.0	7.5	10.0	11.7
Temperature (°C)	C	21.6	25	30	35	38.4

Be that as it may, it is pertinent to understand of some key terms before beginning the discussion on the application of response surface in the optimization of media compositions and fermentation conditions for surfactin and biomass production. The formula and definition of each term that applied are presented in Appendix D.

3.2.4 Analysis of surfactin

Sample broth was withdrawn for about 20 ml and then centrifuged (10,000*g*, 20 min, 4 °C) in a centrifuge manufactured by Hanil Science Industrial to remove the biomass. Afterwards, the cell-free supernatant was subjected to an acid precipitation by adjusting the pH to 2.0 with 6 M HCl and left overnight at 4 °C (Gong et al., 2009). Finally, the

precipitate was collected by centrifugation (10,000g, 20 min, 4 °C) and dissolved in 2 mL of methanol. Surfactin chromatogram and concentration were determined by using reverse phase HPLC (see Appendix B). The system used was an Agilent 1260 Infinity Quaternary LC System (USA) with a C18 column (5 µm, Merck). The mobile phase consisted of 20% of 3.8 mM trifluoroacetic acid (TFA) and 80% acetonitrile. All solvents used were of HPLC grade. 10 µl of sample volume was injected into the HPLC and the elution rate was set at 1 $mLmin^{-1}$. The absorbance of the eluent was monitored at 205 nm. The retention time and peak were compared with the standard surfactin from Sigma (St Louis, MO). Finally, surfactin production was measured and quantified by a standard calibration curve (see Appendix C).

3.2.5 Analysis of biomass

In this research, biomass concentration was determined by measuring the cell dry weight. At the end of fermentation, biomass was harvested from the crude fermentation broth by centrifugation at 10,000g for 20 min. The latter was then dried to constant weight at 60 °C for sufficient time in laboratory vacuum ovens. Ultimately, the dry weight of the biomass was then measured until a constant weight was achieved and later recorded.

3.2.6 Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) was utilized to determine the molecular mass of HPLC purified isoforms. 0.5 µL of matrix was fixated to the sample spot that contains a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in water and 0.3 mg/ml α-cyano-hydroxycinnamic acid in acetone:ethanol (2:1, v/v). After proper mixing, the sample was spotted onto an anchor chip position, dried and set inside the sample cabinet of AB SCIEX TOF/TOF™ 5800 System (AB SCIEX, Framingham MA).. The molecules were separated according to their mass, accelerated by a voltage of 25 kV and were identified by the ion detector set in reflector mode.