ELISA reader. Each sample from the test wells were streaked on PDA plates. Monitoring was conducted after incubation. MFC values were transport as lower sample that did not have any clear colony on the agar plate after incubation period of concentration.

CHAPTER III

MATERIAL AND METHODS

3.1 Surfactin production using local isolate of *B. subtilis* MSH1

3.1.1 Source

The bacterial strain *B. subtilis* MSH1 was isolated from oil contaminated soils collected in Kajang (Selangor, Malaysia) (Shannaq & Isa 2013).

3.1.2 Media and Preparation of Inoculum

Two loopfuls of fresh single pure colonies of *B. subtilis* MSH1 was transferred aseptically from nutrient agar plate into 50 ml of (BHI) with 2% (w/v) glucose in 250 ml Erlenmeyer flasks. The initial pH of inoculums were adjusted to 7.0 the inoculums then incubated in incubator shaker at 150 rpm for 24 h at 30 °C. Two ml of the culture was then inoculated into 250 ml of Cooper's media which had the following composition: 4% glucose (40 g/L), KH_2PO_4 = 30 mM, Na_2HPO_4 = 40 mM, NH_4NO_3 = 50 mM, $MgSO_4$ = 0.8 mM, $FeSO_4$ = 4 µM, $CaCl_2$ = 7 µM and EDTA = 4 µM (Sen & Swaminathan, 1997) in 500 ml Erlenmeyer flasks.

3.1.3 Extraction of surfactin

To extract surfactin using method proposed by Abushady *et al.,* (2005). Isolation of crude biosurfactant from the cell free broth of growth culture. The bacterial cells are disposal from biosurfactants containing culture broth through centrifugation at 10,000 rpm at 4 °C for 20 min. The supernatant deposition overnight at 4 °C by adding concentrated HCl in order to achieve a pH of 2.0. Grey white pellets forms by

sedimentation are collected through centrifugation at 10,000 rpm at 4 °C for 20 min. The pellets are dissolved with methanol and then filtered through 0.45 µm nylon membrane filter.

3.1.4 Quantitative Analysis of surfactin

High-performance liquid chromatography (HPLC) is a technique in analytic chemistry utilized to separation the elements in a blend, to identify each component, and to determine the size of each component. this technique depend on the work of the pumps to passing a pressurized liquid solvent which contains the sample mix through a column filled with a solid adsorbent material. Interacts each element in the sample little differently with the adsorbent material, causing different flow rates for the various ingredients and leading to the separation of the components as they flow out the column (Gerber *et al.*, 2004).

We can be described chromatography as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbents, causing to the separation of the sample components. Usually a granular material made of solid particles is The active component of the column, the sorbent, (e.g. silica, polymers, etc.), 2–50 micrometers in size (Xaing & Lee 2006). Different degrees of interaction with the sorbent particles leading to separated components of the sample mixture from each other.

Usually, the pressurized liquid is a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is named as a "mobile phase". Formation of The pressurized liquid and temperature play a major role in the separation process through the influence on the interactions taking place between sample components and sorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination thereof (Horváth & Lipsky 1967).

3.1.5 Surfactin Calibration Curve

Standard solution was prepared in several concentrations (100mg/L, 200mg/L, 300mg/L, 500mg/L, 600mg/L and 800mg/L) from the stock surfactin solution. Surfactin standard of various concentrations were prepared with methanol HPLC grade and were analyzed using HPLC for construction of calibration curve.

3.2 Antifungal activity of surfactin produced by *B. subtilis* MSH1

3.2.1 Preparation of Fungi

A. niger, *C. gloeosporioides*, *C. albicans* ATCC 1405, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were provided by Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia. Test microorganisms were prepared by inoculated on plates with potato dextrose agar for 7 days.

3.2.2. Determination of Fungi Growth

Determination of fungal concentration was referred to the method proposed by wer & Chu, (1998). Cellular growth was expressed in term of optical density at 600 nm (OD600) measured by Bio-spectrophotometer (Eppendorf, Germany).

3.3. Well Diffusion Method

Due to increased resistance of some fungal strains pathogenic to a number of antibiotics has been proposed to use the traditional allergy testing for anti-fungal and considered well diffusion of ways that contribute to the detection of new compounds characterized by antifungal way. The well diffusion test is simple and easy for reproduction, low-cost and

easy both to reading and interpreting, and can be an alternative way to anti the drug sensitivity test of fungi, in labs with few resources.

One mL saline solution was poured on each of fungi colonies and the spores removed with the aid of a loop. The suspension was collected and transferred to a sterile tube. Wells of 5 mm diameter and 5 mm depth was made in the solidified agar using a sterile borer. The agar well diffusion technique was utilized for the identifying antifungal activity of the recovered surfactin. The numbers of fungi cells were determined in a neubauer chamber and the concentration adjusted to 5×103 cells; per millilitre. One millilitre of this suspension was added to 99 mL of sterile PDA to 45 °C homogenized with a sterilized glass bar and transferred to a plate (Leifert *et al.*, 1995). Various concentrations of surfactin (50, 100, 150, 200, 250 mg/L) were separately place in the wells which were made with the inclusion of sterile cork borer. Plates were incubated at 30 °C for 5 days and then the zone of inhibition was observed. The sensitivity of the test organisms to the extracts were determined by measuring the diameters of inhibition zone surrounding the wells. The diameters of inhibition zones were measured with a ruler in millimeter (mm).

3.4 Determination of the Minimal Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibit the visible growth of a microorganism after overnight incubation. (MIC) are important in diagnostic laboratories to identified the capability of microorganisms resistant to antimicrobial factor also contributes to the monitoring and control the new antimicrobial activity agents (Andrews, 2001). (MIC) is considered one of the most utilized methods of measuring the efficiency of antiviral agents for the growth of pathogens is also utilized in identifying the capability of pathogens resistant to antibiotics (Turnidge *et al.*, 2003).

The fungal strains of *Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans* ATCC 1405, *Candida tropicalis* ATCC750, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC are seed on 96-well plates at a density of 2 x 10^5 cells per well in a volume of 100 of malt extract broth. A 100 µL of surfactin (50, 100, 150, 200, 250 mg/L) add to the wills which contains fungal cells and incubate for 24 h at 28 °C. The optical density of each well was measured at 580 nm by a microtiter ELISA reader. The MIC is the lowest concentration of surfactin needed to stop the microbe from reproducing.

3.5 Determination of Minimum Fungal Concentration (MFC)

The minimum fungal concentration (MFC) is the lowest concentration of an antifungal agent necessary to minimize the growth of fungi. It can be identified from broth dilution minimum inhibitory concentration (MIC) through transport to agar plates that do not include the test agent. The MFC identified by determining the lowest concentration of antifungal agent that reduces survival the inoculum fungal by ≥99.9%. Usually antifungal agents are considered as fungal if the MFC is not more than four times the MIC. Resulting to the utilized MFC to test colony-forming units as a proxy measure of fungal survival, it can be confounded by antifungal agents who leads collects fungal cells. Examples of antifungal agents which do this include flavonoids (Cushnie *et al.*, 2007) and peptides (Suarez *et al.*, 2005).

Determine the minimum concentration of fungal were conducted (MFC) value based on the proposed method by Igbinosa and Okoh (2009) with a few modifications. In this method, fungal strains of *Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans* ATCC 1405, *Candida tropicalis* ATCC750, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC are seed on 96-well plates at a density of 2 x 10^5 cells per well in a volume of 100 of malt extract broth. A 100 µL of surfactin (50, 100, 150, 200, 250 mg/L) add to the wills which contains fungal cells and incubate for 24 h at 28 °C. The optical density of each well was measured at 580 nm by a microtiter