CHAPTER V

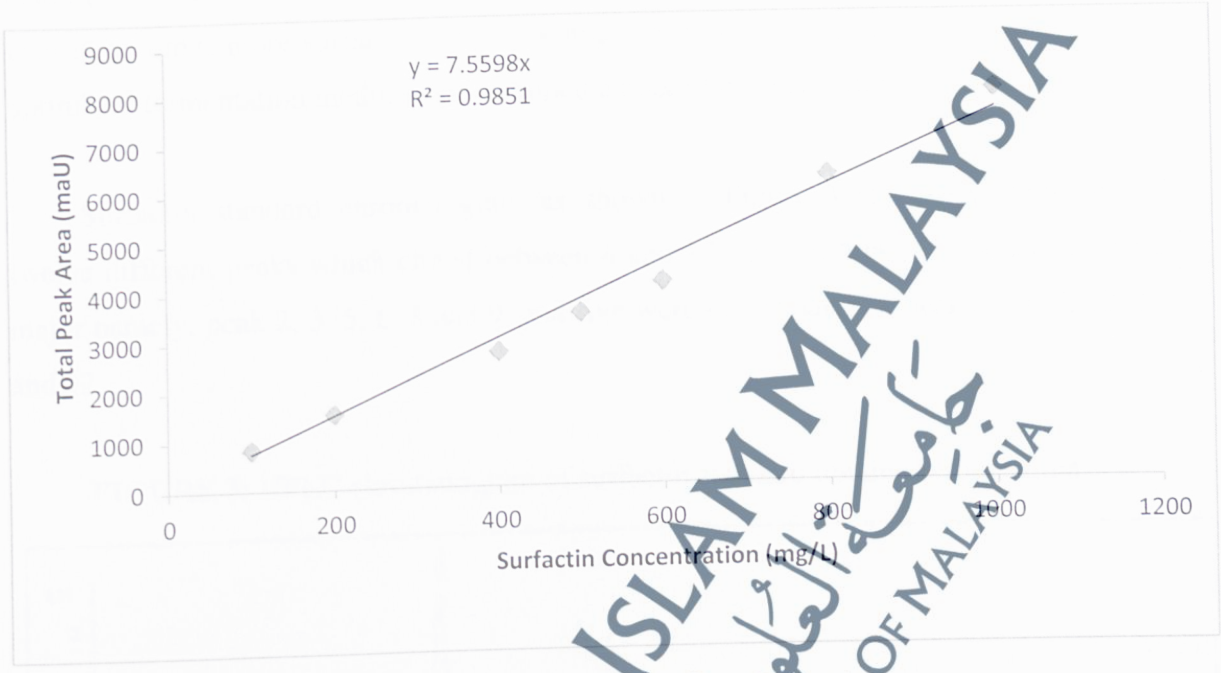
RESULTS AND DISCUSSION

4.1 Surfactin production

Surfactin concentration has been measured through utilizing (HPLC) (Agilent Technologies, 1200 series, USA) Equipped with a C-18 column (Agilent Zorbax Eclipse C18, 250 mm × 4.6 mm, 5µm) and disclosed at 205 nm with a Variable Wavelength Detector (VWD). The system was run at a flow rate of 1.5 ml/min with a mobile phase made up from 3.8 mM trifluoroacetic acid (TFA) in 80% acetonitrile under isocratic condition.

The peaks identified from various concentrations of surfactin standard were summed to produce Total Peak Area (TPA) which was later used to construct a calibration curve for surfactin identification and quantification as shown in Figure 2. A linear equation was obtained with $y = 7.5598x$ and the regression of coefficient (R^2) obtained was 0.9851.

FIGURE 2: Calibration curve of surfactin standard



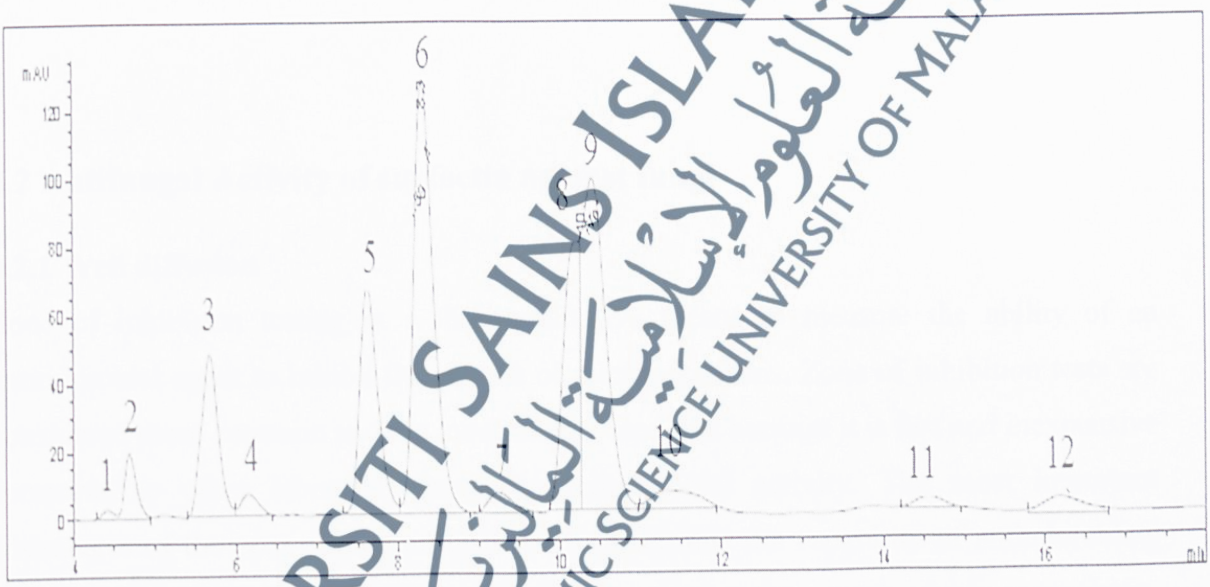
HPLC assay used in this research for surfactin determination and quantification was hypersensitive and replicable. The identification and quantification of surfactin is complicated since surfactin has many of isoforms (Wei & Chu, 2002). An earlier study by Wei *et. al,* (2003) demonstrated that surfactin has six isoforms; others reported that so far at least nine different surfactin isoforms have been determined (Abdel-Mawgoud *et al.,* 2008; Lin & Jiang, 1997). These isoforms vary in the chain length of β-hydroxy fatty acid, which is the most common C13 to C15 (Vater *et al.,* 2002). For that, HPLC analysis was carried out in this study to identify and measure surfactin presence in the crude fermentation broth.

In this study the concentration of surfactin produced by *B. subtilis* MSH1 after 96 h of fermentation was found 105 mg/mL, whereas Shannaq, *(2014)* reported *B. subtilis* MSH1 produced 83 mg/mL. Arima *et al.,* (1968) managed to produce surfactin with concentration of 50-100 mg/L after 24 h of fermentation. Cooper *et al.,* (1981) using

mineral salts medium as fermentation media which resulted in an improved surfactin yield of 780 mg/L with continuous product removal and metal cation addition. Mulligan *et al.,* (1989) discovered an ultraviolet mutant of *B. subtilis* ATCC 21332 which produced over three times more surfactin with 1124 mg/L. Sen & Swaminathan (1997) utilized an optimized fermentation medium and obtained a maximum surfactin yield of 760 mg/L.

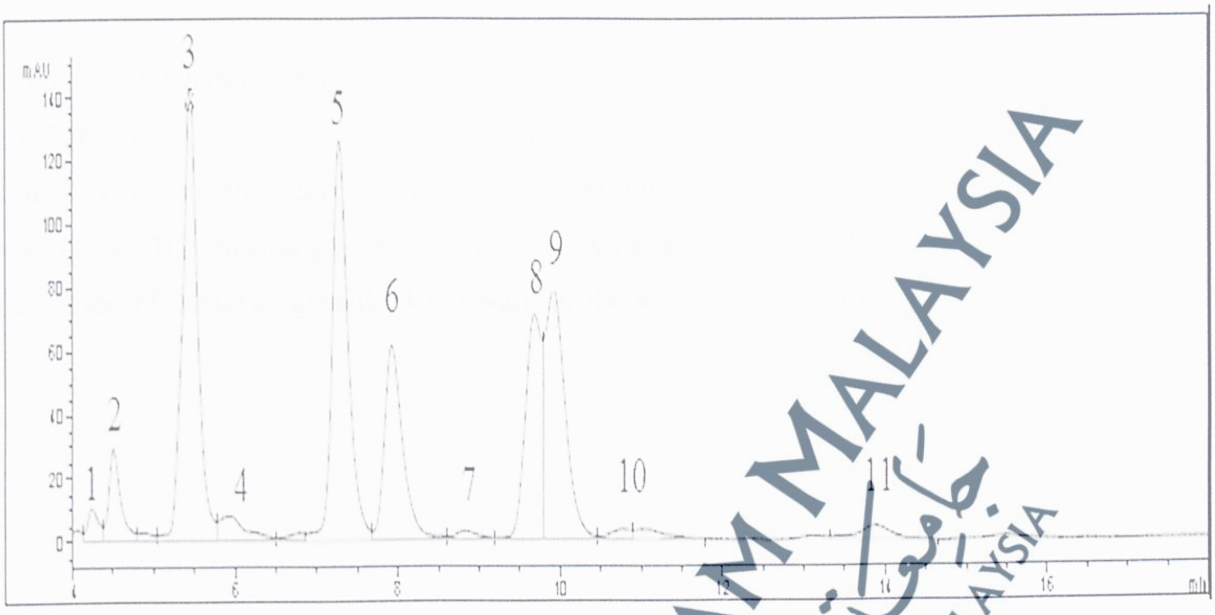
Surfactin standard chromatogram as shown in Figure 3 shows the presence of twelve different peaks which eluted between 4 and 17 min. Of these ten peaks, six were major namely, peak 2, 3, 5, 6, 8 and 9, and five were minor namely, peaks 1, 4, 7, 10, 11 and 12.

FIGURE 3: HPLC chromatogram of surfactin standard obtained from sigma.



Surfactin chromatogram in Figure 4 show *B. subtilis* MSH1 produced eleven different peaks which eluted between 4 and 15 min. Of these peaks, six were major namely peak 2, 3, 5, 6, 8 and 9, and five were minor namely peak 1, 4, 7, 10 and 11. In addition, the figure shows that all peaks were well separated except for peak numbers 8 and 9, which differed in their retention times by less than 0.5 min and appeared somewhat merged.

FIGURE 4: HPLC chromatogram of surfactin produced by *B. subtilis* MSH1.



4.2 Antifungal Activity of surfactin against fungi

4.2.1 Well diffusion

Zone of inhibition testing is a fast, qualitative means to measure the ability of an antimicrobial agent to inhibit the growth of microorganisms. Zone of inhibition tests are considered more common and the most widely used that because it is fast and inexpensive compared to other laboratory tests for antimicrobial activity. The most important advantages of the zone inhibition tests are to determine the capability of antimicrobials and help them to blend into the water in order to prevent the growth of microorganisms. Through the use of this test, we can examine a number of samples and determine the characteristics of each sample if their have anti-microbial activity. This test also helps to examine and study many types of microbial products. There are many tests that have the ability to configure inhibition zone such as liquids, coated antimicrobial surfaces, and antimicrobial-impregnated solid products.

The antifungal activity of surfactin produced by *B. subtilis* MSH1 were studied in different concentrations (50, 100, 150, 200 and 250 mg/l) against six pathogenic fungal strains, (*Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans* ATCC 1405, *Candida tropicalis* ATCC750, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258). By comparison, streptomycin (positive control) its concentration was (25mg/l) . Antifungal possibilities of extracts were evaluated in terms of zone of inhibition of bacterial growth. The result of the antifungal activities are showed in Table 1.

TABLE 1 : Diameter of inhibition zone (mm) for antimicrobial test of surfactin by agar-well diffusion test

Concentration of test samples (mg/ml)	Test fungi					
	A. niger	*C. gloeosporioides*	*C. albican*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*
250.00[a]	15 ±0.10[b]	14 ±0.10	13 ±0.10	11.5 ±0.10	10 ±0.10	11 ±0.10
200.00	13 ±0.10	11 ±0.10	11 ±0.20	9 ±0.10	8 ±0.20	8 ±0.25
150.00	10 ±0.10	8.5 ±0.10	9.5 ±0.10	5.5 ±0.10	4.5 ±0.10	5.0 ±0.10
100.00	5.5 ±0.10	5.0 ±0.10	6.0 ±0.10	3.5 ±0.10	2.0 ±0.10	1.5 ±0.17
50.00	2.5± 0	2.0± 0	3.0 ± 0	1.5 ± 0	1.0 ± 0	0.5 ± 0
S10[c]	33.0 ± 0	25.0 ± 0	29.0 ± 0	32.0 ± 0	24.0 ± 0	25.0 ± 0

[a] = Concentration of test sample (mg/ml)

[b] = Diameter of inhibition zones (mm)

[c] = Streptomycin sulfate (25mg/l)

The antifungal activity of surfactin increased linearly with increase in concentration of surfactin (mg/l). The results detected that in the extracts for fungal activity, *A. niger, C. gloeosporioides*, *C. albicans* shows good result as comparison with *C. tropicalis*, *C. parapsilosis* and *C. krusei*. The growth inhibition zone measuring ranged from 0.5 to 15 mm for fungal strains. Size of the zone of inhibition proportional directly proportional to the effectiveness of the activity factor anti-microbe is the largest area thus inhibiting means that the antimicrobial more effectively.

4.2.2 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungal Concentration (MFC) Values of Surfactin

The antifungal activity of surfactin produced by *B. subtilis* MSH1 was assessed by determination of MIC and MFC values. Table 2 shows MIC of surfactin produced by *B. subtilis* MSH1 was found to be at 100 mg/L and 150 mg/L against *Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans, Candida tropicalis, Candida parapsilosis* and *Candida krusei*. The growth of *Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans , Candida tropicalis , Candida parapsilosis* and *Candida krusei* were completely inhibited with treatment of 250 mg/L, 200 mg/L and 150 mg/L of surfactin, respectively as shown in Table 3, and these concentrations were determined as the minimum bactericidal concentration (MFC).

TABLE 2: The MIC value of surfactin

Test fungi	MIC (mg/ml)
A. niger	100.0
C. gloeosporioides	100.0
C. albican	100.0
C. tropicalis	150.0
C. parapsilosis	150.0
C. krusi	150.0

TABLE 3: The MFC value of surfactin

Test fungi	MFC (mg/ml)
A. niger	100.0
C. gloeosporioides	100.0
C. albican	150.0
C. tropicalis	150.0
C. parapsilosis	150.0
C. krusi	150.0

The antifungal sensitivity of the extract surfactin and their potency were assessed quantitatively by determining the MIC, MFC as given in Tables 2 and 3. In this study, the growth of fungal was remarkably inhibited by the surfactin especially high concentrations of surfactin (250mg\l, 200 mg\l, and 150 mg\l). this is in comparison with the low concentrations of surfactin (100 mg/l and 50 mg/l) growth of fungal was significantly in *C. tropicalis* , *C. parapsilosis* and *C. krusei* compare by *A. niger, C. gloeosporioides, C. albicans.* Despite the low concentration of surfactin , but it was the inhibition of growth it. To ensure the result, I took a loop and deep into each sample. Then streak onto agar and incubate for 24 hours again for MFC to look at the regrowth of the microbe.

If the MIC/MFC value is less than or equal to 4 (≤4) the strains is considered to be susceptible, while if the ratio is greater than 4 (>4) then the fungi is considered to be tolerant. The MFC/MIC value of surfactin towards six strains of fungal tested (*A. niger, C. gloeosporioides, C. albicans, C. tropicalis, C. parapsilosis* and *C. krusei.*) is less than or equal to 4 (≤ 4) as summarized in Table 4. It is showed that these six fungal strains were sensitive to the surfactin.

TABLE 4: The MIC, MFC and ratio MFC/MIC values of surfactin

Test fungi	MIC (mg/ml)	MFC (mg/ml)	MFC/MIC[a]
A. niger	100.0	100.0	1
C. gloeosporioides	100.0	100.0	1
C. albican	100.0	150.0	1.5
C. tropicalis	150.0	150.0	1
C. parapsilosis	150.0	150.0	1
C. krusi	150.0	150.0	1

MFC/MIC[a] = Ratio value of MFC over MIC

Results obtained in this study show *B. subtilis* MSH1 were capable to producing surfactin which were bacteriostatic and bactericidal against the six pathogenic funguses tested. Evaluation on the effectiveness of antibacterial agents depends on the mechanism of their activity, which involving the inhibition of cellular processes such as expression of genes; synthesis of vital cellular biomolecules and their transport. In addition, the force of antifungal activity also depends on the sensitivity of the fungal strains towards specific types of antifungal.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Objectives of this study are the production of material surfactin of local isolation of *B. subtilis* MSH1 and then prove that has the antifungal activity. This study showed that local isolation of *B. subtilis* MSH1 has the capability to production surfactin at 30 ^{o}C in a mineral medium contains 4% of (w/v) glucose. In this study also the concentration of surfactin produced by *B. subtilis* MSH1 after 96 h of fermentation was found 105 mg/ml. Antifungal activity was confirmed by surfactin and the results revealed the high concentration of surfactin are the most effective inhibitor for the mycelia growth of *Aspergillus niger, Colletotrichum gloeosporioides, Candida albicans, Candida tropicalis, Candida parapsilosis* and *Candida krusei* this is in comparison with the low concentrations of surfactin. The finding of the present investigation could be an important step towards the possibilities of using surfactin as antimicrobial because its true antibiotic nature has a very significant antibacterial characteristic.

5.2. Recommendations

Based on the findings of the study, the following are recommended for future study on effects of surfactin production by adding various concentration of manganese and glucose to fermentation media, and investigation antiviral and hemolytic activities of surfactin isoforms as well as application of surfactin for enhanced biodegradation of diesel-contaminated water and soil.