CHAPTER IV

RESULTS AND DISCUSSIONS

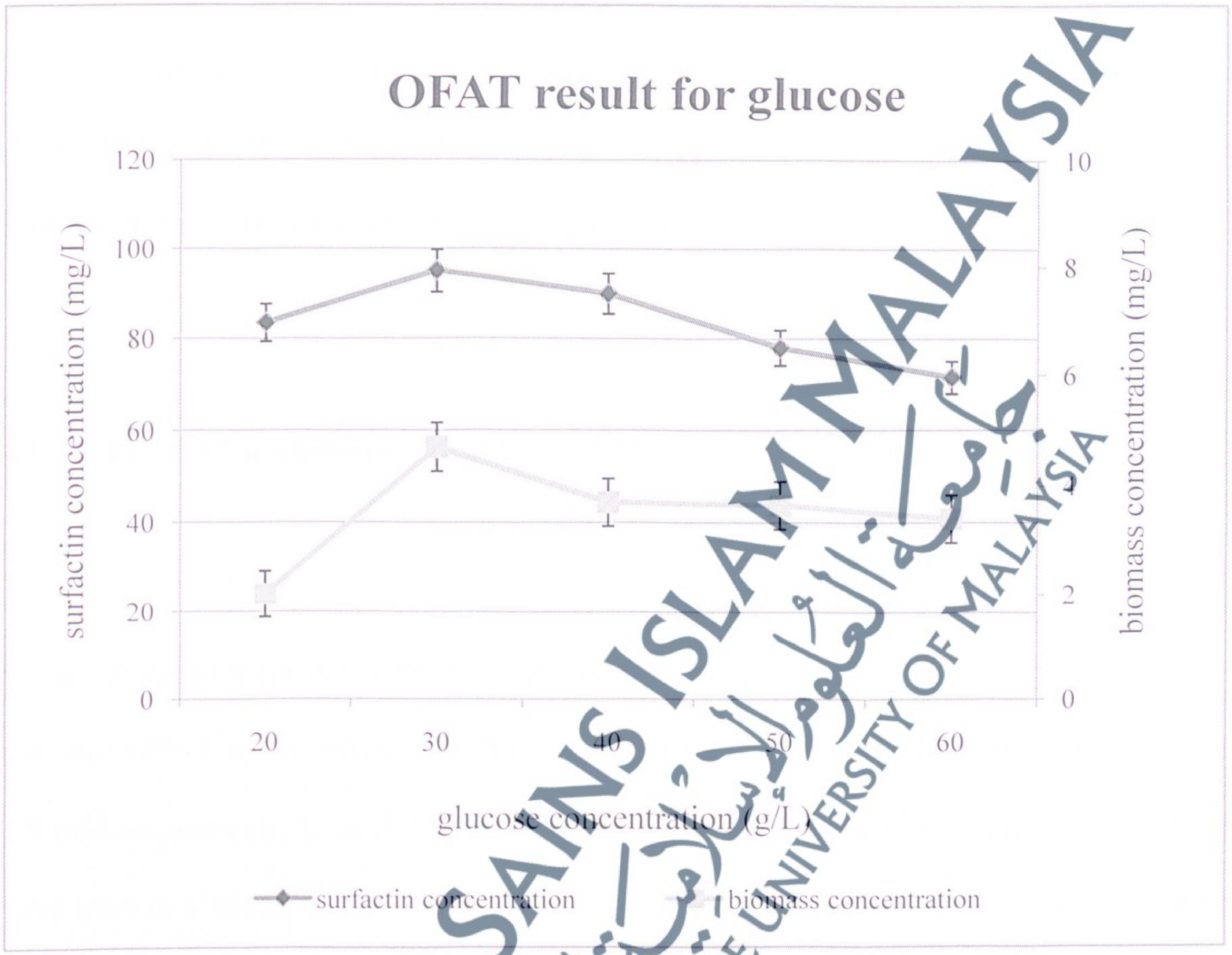
4.1 OFAT optimization study for media composition

Prior to RSM optimization experiment, OFAT technique was employed to determine the effect and desirable ranges of media composition toward surfactin production. The experiments was started by varying glucose concentration, and then followed by ammonium nitrate, ferrous sulfate and manganese sulfate.

4.1.1 Effect of glucose

Glucose was selected as an energy source for bacterial growth as it is a simple sugar in the form of monosaccharide. By using glucose as the main media component, more surfactin is expected to yield rather than its counterparts which are sucrose and glycerol (Onwosi and Odibo, 2012). Figure 3 shows that the production of surfactin and biomass was affected by various concentrations of glucose in Cooper's media.

Figure 3: Effect of various concentrations of glucose on the yield of surfactin and biomass produced by *B. subtilis* 3M for 96 h at 30 °C with addition of 5% inoculum



Various concentrations of glucose in the range of 20 to 60 g/L were supplied in Cooper's media to determine the optimal glucose concentration needed for enhancement of surfactin and biomass. In the meantime, other important variables were fixed at the following concentrations: 0.05 M NH_4NO_3, 4 µM $FeSO_4$ and 1.5 mM $MnSO_4$. The highest production of surfactin and biomass was obtained at 30 g/L of glucose concentration with the concentration of 95 and 4.7 mg/L respectively. However, surfactin and biomass

production decreased significantly at the concentration of glucose more than 40 g/L, indicating a possible inhibitory effect of glucose at high concentration. This was due to the role played by Crabtree Effect that occurred during high concentration of glucose in the media. Availability of extra pyruvate, formed from glycolysis of glucose prevents the slight decline in the endogenous oxygen consumption rate, and thus reduces surfactin production by *B. subtlis* bacteria (De Deken, 1966; Pfeiffer & Morley, 2014).

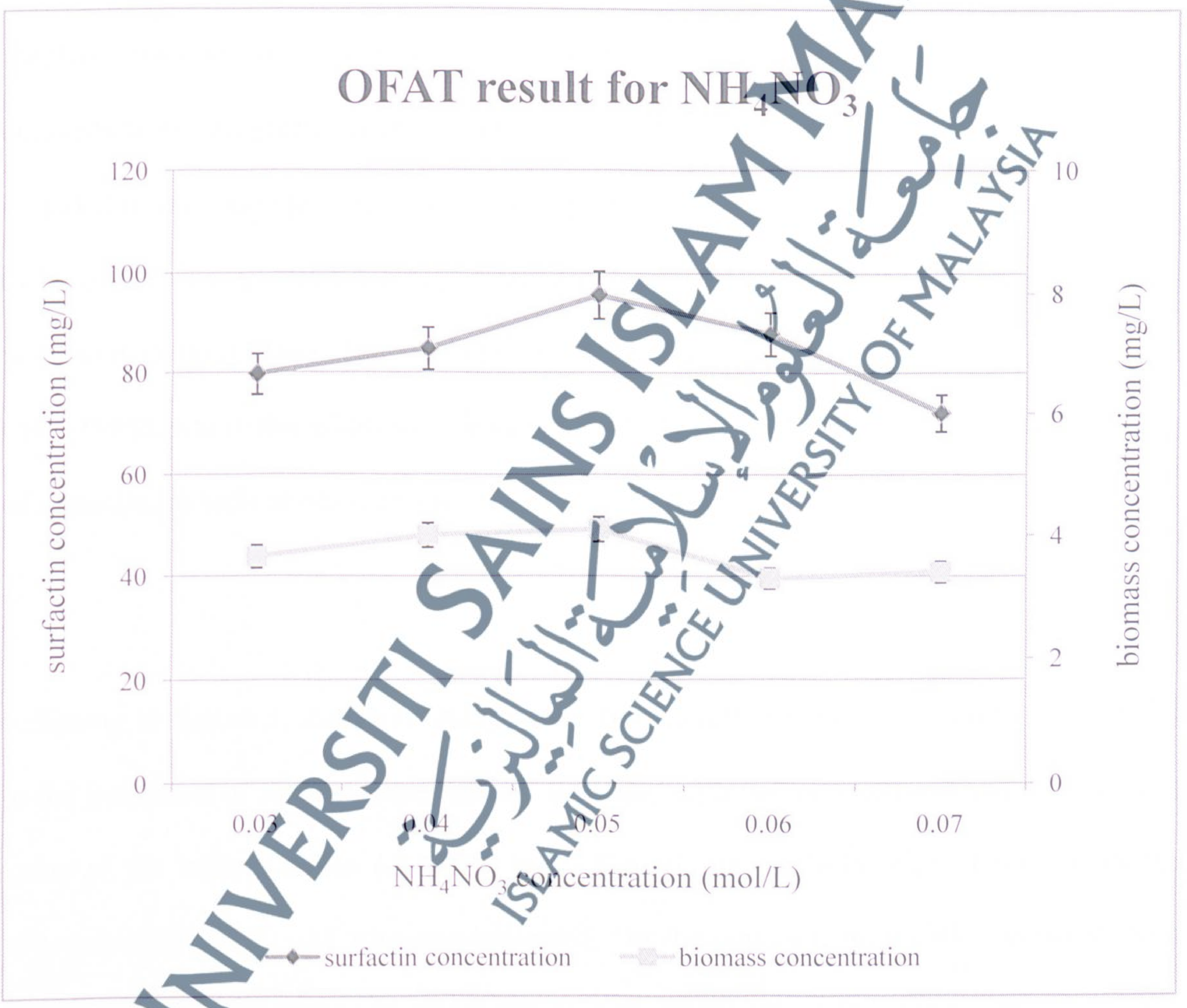
4.1.2 Effect of ammonium nitrate (NH_4NO_3)

In this study, glucose concentration was fixed at an optimal value of 30 g/L according to previous OFAT result, whereas $FeSO_4$ and $MnSO_4$ concentrations were fixed at 4 µM and 1.5 mM respectively. Five different NH_4NO_3 concentrations ranging from 0.03 to 0.07 M were used to evaluate the effect of NH_4NO_3 concentrations in Cooper's media on surfactin and biomass production after 96 h of fermentation.

Referring to Figure 4, increasing NH_4NO_3 concentration from 0.03 to 0.05 M increased surfactin and biomass production significantly, in which the highest concentration of 95.6 and 4.1 mg/L respectively were obtained for NH_4NO_3 with the concentration of 0.05 M. According to Ye et al. (2008), supplementation of nitrogen source in the media is

necessary for fast microorganism growth. This is also highly beneficial for long time accumulation of surfactin during fermentation process of *B. subtilis*.

Figure 4: Effect of various concentrations of NH_4NO_3 on the yield of surfactin and biomass produced by *B. subtilis* 3M for 96 h at 30 °C with addition of 3% inoculum

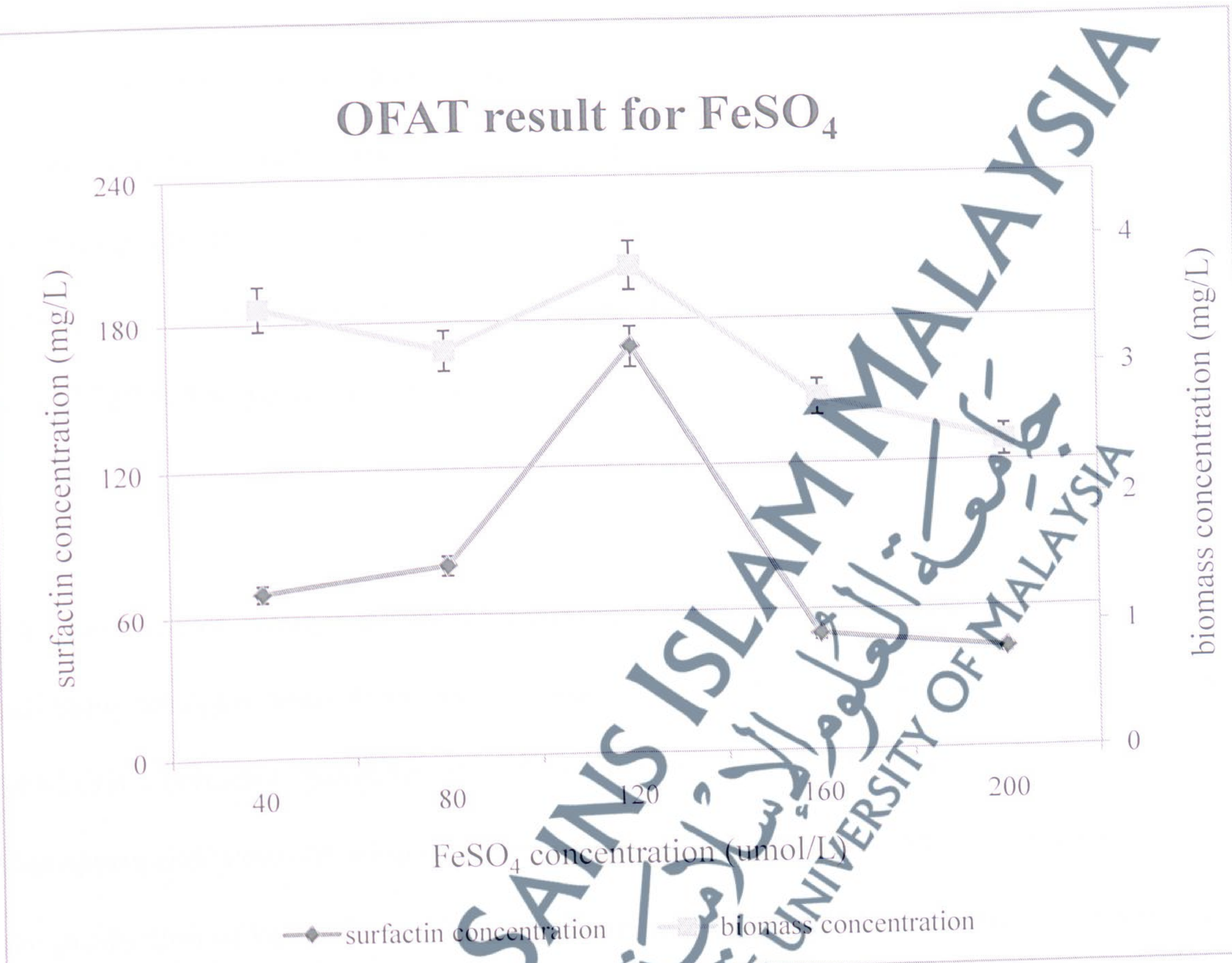


4.1.3 Effect of ferrous sulfate ($FeSO_4$)

Figure 5 depicts the effect of various $FeSO_4$ concentrations on surfactin and biomass production. Glucose and NH_4NO_3 concentrations were fixed at the optimal value of 30 g/L and 0.05 M respectively according to previous OFAT result, whereas $MnSO_4$ concentration was fixed at 1.5 mM. In the meantime, five different $FeSO_4$ concentrations ranging from 40 to 200 µM were used to evaluate the effect of various $FeSO_4$ concentrations on surfactin and biomass production. It was noted that 4 µM was not included in the range (as previous OFAT optimization) since an appropriate range needed to be established to show the significance of various $FeSO_4$ concentrations on surfactin and biomass production. The explanation for this adjustment was suggested by Wei and Chu (1998), where the addition of iron from 4 µM to 4 mM could induce overproduction of surfactin, as well as biomass growth.

Referring to Figure 5, surfactin and biomass concentration increased steadily and parallel to the increment of $FeSO_4$ concentration. Both surfactin and biomass reached their highest value at the concentration of 168.5 and 3.8 mg/L respectively when $FeSO_4$ with the concentration of 120 µM was supplemented. On the contrary, high $FeSO_4$ concentration (120 to 200 µM) caused rapid acidification of the broth, hence induced the disappearance of surfactin as the pH dropped below 5.0 (Wei and Chu, 1998). From the result, it is proved that appropriate amounts of iron are required to create suitable growth environment for *B. subtilis*.

Figure 5: Effect of various concentrations of $FeSO_4$ on the yield of surfactin and biomass produced by *B. subtilis* 3M for 96 h at 30 °C with addition of 5% inoculum

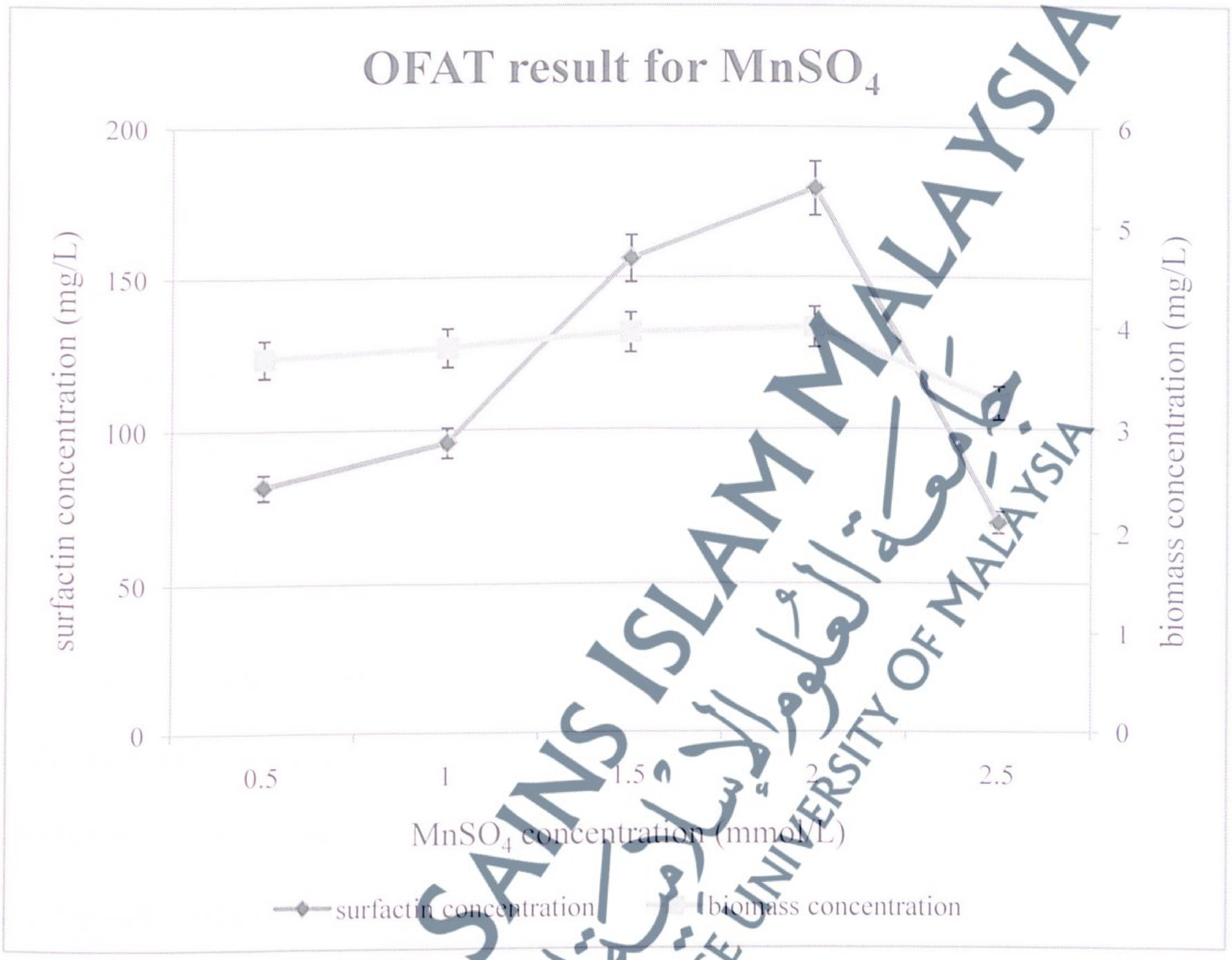


4.1.4 Effect of manganese sulfate ($MnSO_4$)

Various concentrations of $MnSO_4$ ranging from 0.5 to 2.5 mM were supplemented in Cooper's media to determine the optimal concentration needed to enhance surfactin and biomass production as illustrated in Figure 6. At the same time, glucose, NH_4NO_3 and $FeSO_4$ concentrations were fixed according to their optimal concentration of 30 g/L, 0.05 M and 120 µM respectively based on previous OFAT optimization result.

The presence of manganese in the medium is crucial since it supports cell growth by affecting nitrogen utilization and K^+ ions uptake of *B. subtilis* cells (Wei and Chu, 2002).This behavior stimulates *B. subtilis* to yield high production of surfactin provided that appropriate concentration of manganese is supplied into the media. Based on Figure 6, the production of surfactin and biomass increased rapidly with the increase of manganese sulfate concentration from 0.5 to 2.0 mM. The highest amount of surfactin and biomass production was obtained at 179.4 and 4.0 mg/L respectively when the concentration of $MnSO_4$ was at 2.0 mM. Further increase of the latter after 2.0 mM, however, resulted in the reduction of the formers production.

Figure 6: Effect of various concentrations of $MnSO_4$ on the yield of surfactin and biomass produced by *B. subtilis* 3M for 96 h at 30 °C with addition of 5% inoculum



4.2 RSM optimization study for media composition

In our previous study, the medium composition was optimized by one-factor-at-a-time (OFAT) optimization, which involves changing one independent variable while keeping other factors constant. Based on the result, glucose, ammonium nitrate, ferrous sulfate and manganese sulfate concentrations play an important role on surfactin and biomass production. Therefore, these four factors were selected for further statistical optimization of both responses by using RSM.

The effect of various concentrations of glucose, ammonium nitrate, ferrous sulfate and manganese sulfate on both surfactin and biomass production was studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$), where $\alpha = 2^{n/4}$ and n is the number of parameters, whereas 0 corresponds to the central level that was selected from the preliminary work.

4.2.1 Analysis on the production of surfactin and biomass

A statistical software package Design Expert 7.1.6 was used for regression analysis of experimental data and plotting response surfaces. This application estimate results in an empirical relationship between the values of surfactin and biomass, as well as the process variables. Through multiple regression analysis on the experimental data, relative surfactin

and biomass yield as functions of the test variables were represented by the following second-order polynomial equations:

Surfactin concentration =

$$197.67 + 4.93A + 1.39B + 7.46C + 8.97D + 1.46AB + 9.06AC + 2.50AD - 0.54BC + 5.18BD + 7.13CD - 17.72A^2 - 17.03B^2 - 21.20C^2 - 20.68D^2 \quad (1)$$

Biomass concentration =

$$4.47 + 0.30A + 0.058B + 0.20C + 0.26D - 0.081AB + 0.063AC - 0.11AD + 0.050BC + 6.25e^{-3}BD + 0.14CD - 0.34A^2 + 0.049B^2 - 0.13C^2 - 0.20D^2 \quad (2)$$

Where A, B, C and D are glucose (g/L), ammonium nitrate (M), ferrous sulfate (μM) and manganese sulfate (mM) concentrations respectively.

Table 7: Central composite design arrangement, responses and predicted values for surfactin and biomass yield

Run	A	B	C	D	Surfactin concentration		Biomass concentration	
					Experimental	Predicted	Experimental	Predicted
1	30	0.04	80	1.0	126.8	129.4	3.10	3.13
2	50	0.04	80	1.0	99.1	103.6	4.30	4.15
3	30	0.06	80	1.0	108.3	110.4	3.45	3.47
4	50	0.06	80	1.0	113.7	109.6	3.75	3.81
5	30	0.04	160	1.0	102.6	103.4	3.30	3.21
6	50	0.04	160	1.0	133.0	133.0	4.10	4.12
7	30	0.06	160	1.0	99.4	101.4	3.40	3.40
8	50	0.06	160	1.0	114.9	117.8	4.45	4.34
9	30	0.04	80	2.0	111.1	108.1	3.70	3.75
10	50	0.04	80	2.0	111.6	111.5	3.90	3.99
11	30	0.06	80	2.0	127.1	129.0	3.70	3.76
12	50	0.06	80	2.0	120.0	119.1	4.00	4.03
13	30	0.04	160	2.0	123.8	129.8	4.00	4.02
14	50	0.04	160	2.0	152.5	150.3	4.95	4.86
15	30	0.06	160	2.0	134.0	129.4	4.50	4.59
16	50	0.06	160	2.0	175.5	174.9	4.70	4.76
17	40	0.05	120	1.5	184.2	194.4	4.70	4.68
18	40	0.05	120	1.5	204.2	194.4	4.40	4.68
19	40	0.05	120	1.5	215.7	204.0	4.75	4.50
20	40	0.05	120	1.5	199.9	204.0	4.60	4.50
21	20	0.05	120	1.5	116.9	113.9	2.35	2.27
22	60	0.05	120	1.5	152.5	133.7	3.40	3.46
23	40	0.03	120	1.5	127.2	123.8	4.25	4.32
24	40	0.07	120	1.5	127.7	129.3	4.65	4.56
25	40	0.05	40	1.5	95.5	94.9	3.40	3.31
26	40	0.05	200	1.5	126.0	124.8	4.05	4.11
27	40	0.05	120	0.5	98.5	94.0	2.80	2.92
28	40	0.05	120	2.5	127.2	129.9	4.10	3.96
29	40	0.05	120	1.5	187.8	194.7	3.90	4.24
30	40	0.05	120	1.5	194.2	194.7	4.50	4.24

The mean predicted and observed responses are presented in Table 7. Statistical testing of the model was done using Fisher's statistical test (*F*-test) and *P*-values for analysis of variance (ANOVA). A good *F*-value validated that the factors can explain adequately the variations in the data about its mean. On the other hand, *P*-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. Smaller *P*-values correspond to more significant coefficient (Yu et al., 2009; Huang et al., 2012).

The summary of ANOVA in Table 8 and 9 represents the results of the quadratic response surface model for surfactin and biomass production respectively. From ANOVA, it can be observed that the models for surfactin and biomass production were highly significant as evident from a high *F*-value and a very low *P*-value. The *F*-values for surfactin and biomass production models were 55.17 and 18.88 respectively, and there was only 0.01% chance that a model *F*-value this large could occur due to noise. Since the *F*-value was greater than one, it was more confident that the factors could explain adequately the variation in the data about its mean. Meanwhile, values of "Prob> F" or *P*-value less than 0.0500 indicated that the model terms were considered fit and thus could effectively explain the mutual interactions between the test variables. Among the model terms in this study, A, C, D, AC, BC, CD, A^2, B^2, C^2, D^2 and A, C, D, AD, CD, A^2, C^2, D^2 had very significant influence on surfactin and biomass production respectively.

The "lack of fit" test compares residual error to "pure error" from replicated design points. For the production of surfactin and biomass, the "lack of fit" *F*-value of 0.21 and 0.32 respectively implied that the "lack of fit" was not significant relative to the "pure error". In the case of "lack of fit", non-significant is good and makes the model fit.

Analysis of variance for the regression obtained from this experiment on surfactin and biomass production is shown in Table 10 and 11 respectively. In any statistical analysis, the goodness of fit of the model can be checked by the determination coefficient R^2 and the adjusted R^2 (multiple correlation coefficient R). With that regard, acceptable statistical model value of R^2 should be close to 1.0 and all four factors should be positive and close to each other. The R^2 for the production of surfactin and biomass was found to be 0.9834 and 0.9531 respectively. On the other hand, the adjusted R^2 and predicted R^2 values for the model were 0.9656 and 0.9374 respectively for surfactin production whereas for biomass production, the values were 0.9026 and 0.8238 respectively. According to the result, the model is expected to predict the response more correctly in the present case.

Adequate precision measures signal to noise ratio and a value higher than 4 is desirable. For production of surfactin and biomass, the adequate precision for this model was 21.637 and 17.78 respectively. This high value of adequate precision demonstrated that model is significant for the production of surfactin and biomass. Meanwhile, a low value of coefficient of variation (CV) indicates a very high degree of precision and a good deal of reliability of the experimental values. Low values of CV for surfactin and biomass

production were recorded (4.95 and 4.87 % respectively), which indicated a greater reliability of the experiments performed. As a general rule, a model can be considered reasonably reproducible if its CV is not greater than 10%.

Table 8: Analysis of variance (ANOVA) for all terms in the model for optimization of surfactin production

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	35194.17	14	2513.87	55.17	< 0.0001
A-Glucose	584.11	1	584.11	12.82	0.0034
B-Ammonium Nitrate	46.48	1	46.48	1.02	0.3309
C-Ferrous Sulfate	1335.04	1	1335.04	29.30	0.0001
D-Manganese Sulfate	1929.63	1	1929.63	42.35	< 0.0001
AB	34.22	1	34.22	0.75	0.4019
AC	1314.06	1	1314.06	28.84	0.0001
AD	100.00	1	100.00	2.19	0.1623
BC	4.62	1	4.62	0.10	0.7552
BD	428.49	1	428.49	9.40	0.009
CD	812.25	1	812.25	17.83	0.001
A^2	8609.29	1	8609.29	188.94	< 0.0001
B^2	7954.08	1	7954.08	174.56	< 0.0001
C^2	12332.34	1	12332.34	270.64	< 0.0001
D^2	11729.22	1	11729.22	257.41	< 0.0001
Residual	592.37	13	45.57		
Lack of Fit	247.07	10	24.71	0.21	0.9724
Pure Error	345.30	3	115.10		
Cor Total	36379.71	29			

Table 9: Analysis of variance (ANOVA) for all terms in the model for optimization of biomass production

Source	Sum of Squares	df	Mean Square	F-Value	Prob> F
Model	9.903	14	0.707	18.881	< 0.0001
A-Glucose	2.100	1	2.100	56.064	< 0.0001
B-Ammonium Nitrate	0.082	1	0.082	2.180	0.1636
C-Ferrous Sulfate	0.960	1	0.960	25.624	0.0002
D-Manganese Sulfate	1.602	1	1.602	42.751	< 0.0001
AB	0.106	1	0.106	2.819	0.117
AC	0.063	1	0.063	1.668	0.219
AD	0.181	1	0.181	4.821	0.0469
BC	0.040	1	0.040	1.068	0.3203
BD	0.001	1	0.001	0.017	0.8992
CD	0.303	1	0.303	8.074	0.0139
A^2	3.261	1	3.261	87.035	< 0.0001
B^2	0.066	1	0.066	1.755	0.2081
C^2	0.480	1	0.480	12.813	0.0034
D^2	1.109	1	1.109	29.591	0.0001
Residual	0.487	13	0.037		
Lack of Fit	0.251	10	0.025	0.318	0.9261
Pure Error	0.236	3	0.079		
Cor Total	11.348	29			

Table 10: ANOVA for regression in the optimization of surfactin production

Terms	Value	Terms	Value
Std. Dev.	6.75	R-Squared	0.9834
Mean	136.36	Adj. R-Squared	0.9656
C.V. %	4.95	Pred. R-Squared	0.9374
PRESS	2240.16	Adeq. Precision	21.637

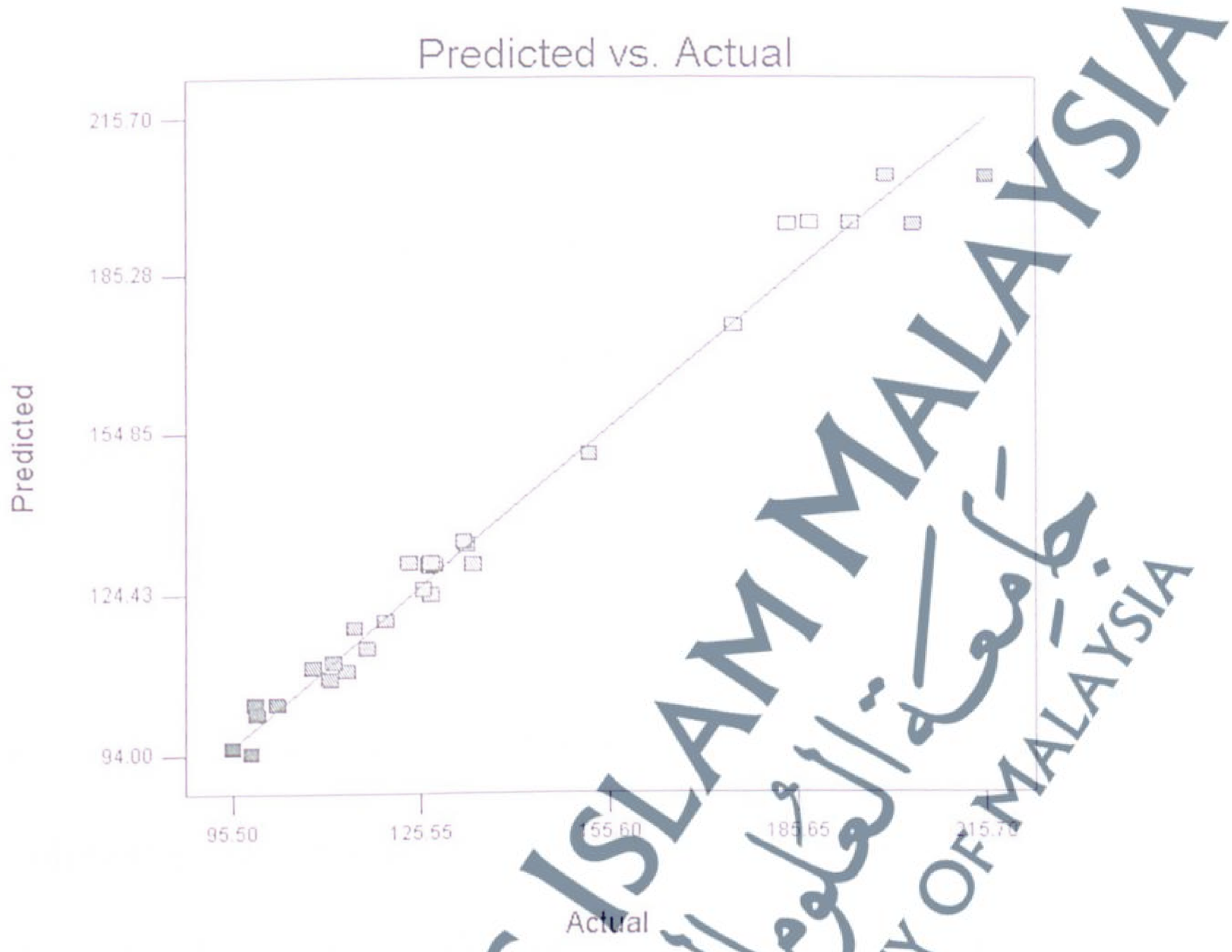
Table 11: ANOVA for regression in the optimization of biomass production

Terms	Value	Terms	Value
Std. Dev.	0.19	R-Squared	0.9531
Mean	3.97	Adj. R-Squared	0.9026
C.V. %	4.87	Pred. R-Squared	0.8238
PRESS	1.83	Adeq. Precision	17.7842

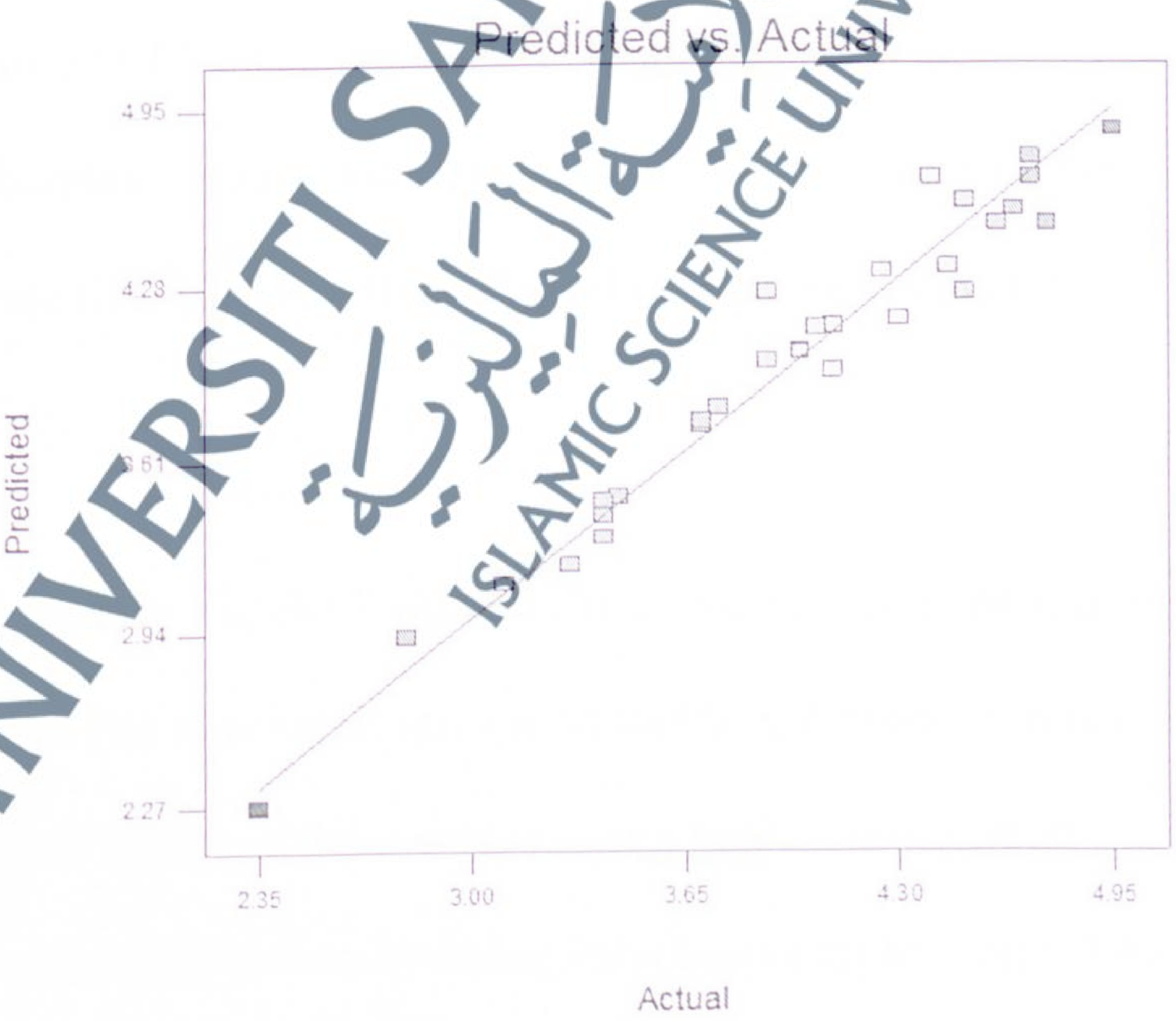
Figures 7 (a) and (b) represent the relationship between the actual and predicted concentration values for surfactin and biomass production respectively that was determined by model equations 1 and 2. From the graph, most points were clearly nearby the line adjustment, meaning that the experimentally determined values were similar to the model. Furthermore, the values of R^2, adjusted R^2 and predicted R^2 mentioned previously were very close to 1.0, which also advocates a high correlation between the observed and predicted values. Thus, the result obtained from the developed model equations can effectively predict surfactin and biomass production.

Figures 7: Comparison between predicted and observed production of (a) surfactin and (b) biomass in mg/L

a)



b)



4.2.2 Combination effects of variables by response surface plots

The 3D response surface plots described by the regression model were drawn to illustrate the effects of independent variables on surfactin and biomass production as the responses. From the 3D response surface plots, the combined effect and interactions between the four variables on the responses can be evaluated and the optimal values of the independent variables can be observed.

Equations 4.1 and 4.2 were then used to facilitate plotting of response surfaces. Two parameters were plotted at one time while another two parameters were maintained at their respective zero levels (coded level: 0). The optimal values for the variables were obtained by moving along the major and minor axis of contour so that the point that yielded the highest surfactin and biomass could be determined. Figures 8 - 13 show the result of surfactin and biomass production affected by various concentrations of glucose (A), ammonium nitrate (B), ferrous sulfate (C) and manganese sulfate (D).

The 3D surface plots in Figures 8 (a) and (b) shows the effect of various glucose (A) and ammonium nitrate (B) concentrations on surfactin and biomass production respectively. As the main carbon source in the media, varying glucose concentration can influence both surfactin and biomass production by either induction or repression (Makkar and Cameotra, 2002). Both figures indicated that the production of surfactin and biomass increased to

optimum concentrations of 197.96 and 4.54 mg/L respectively when the idle concentration of glucose reached the ranges between 40.0 and 44.0 g/L. Afterwards, the production of surfactin and biomass was inhibited due to over-addition of glucose. Surfactin production decreased from 197.76 to 188.73 mg/L with an increased concentration of glucose from 40.76 to 50.0 mg/L. Meanwhile, further increase in glucose concentration higher than 43.3 g/L did not affect biomass production. From Figure 8, the effect of various concentrations of ammonium nitrate in the media on surfactin and biomass production could also be observed.

Figures 9 (a) and (b) show the effect of various glucose and ferrous sulfate concentrations on the production of surfactin and biomass respectively. The figures indicated that both parameters were significant since the contour plot exerted a quadratic effect on surfactin and biomass production. In this case, the production of surfactin and biomass increased gradually from 169.35 to 198.40 mg/L and 4.15 to 4.56 mg/L respectively when the concentration of ferrous sulfate increased to 127 µM and 149 µM respectively. Hence, fermentation by using iron-induced Cooper's media resulted in the significant amount of surfactin recovered. In contrast, the recovery of surfactin and biomass from fermentation broth decreased into approximately 188.24 and 4.43 mg/L respectively when the concentration of ferrous sulfate reached 160 µM. Therefore, it can be concluded that excess dosage of iron may instigate acidogenic fermentation behavior, thus reducing both responses production (Wei et al., 2003). In addition, the influence of glucose concentration on the production of surfactin is also depicted in Figure 9.

Figures 10 (a) and (b) illustrate the interaction of various ammonium nitrate and ferrous sulfate concentrations with the production of surfactin and biomass respectively. When the concentration of ammonium nitrate increased from 0.040 to 0.052 M, surfactin production increased gradually from 179.60 to 197.34 mg/L. Then, the production of surfactin decreased from 197.34 to 186.18 mg/L when the concentration of ammonium nitrate increased from 0.052 to 0.060 M. Ammonium nitrate is an important nitrogen source in medium that influences the production of surfactin. According to the report by Davis et al. (1999), both ammonium and nitrate ions are utilized by *B. subtilis* to enhance surfactin production. The report also mentioned that as anaerobic growth continued after 23 h of fermentation, no further biomass increase was observed. This explains the reason of 3D surface plot that showed an insignificant effect on biomass production despite various ammonium nitrate concentrations were supplied into the media. Furthermore, Figures 8 (b) and 12 (b) that contained ammonium nitrate as one of the parameters also showed the same effect on biomass production.

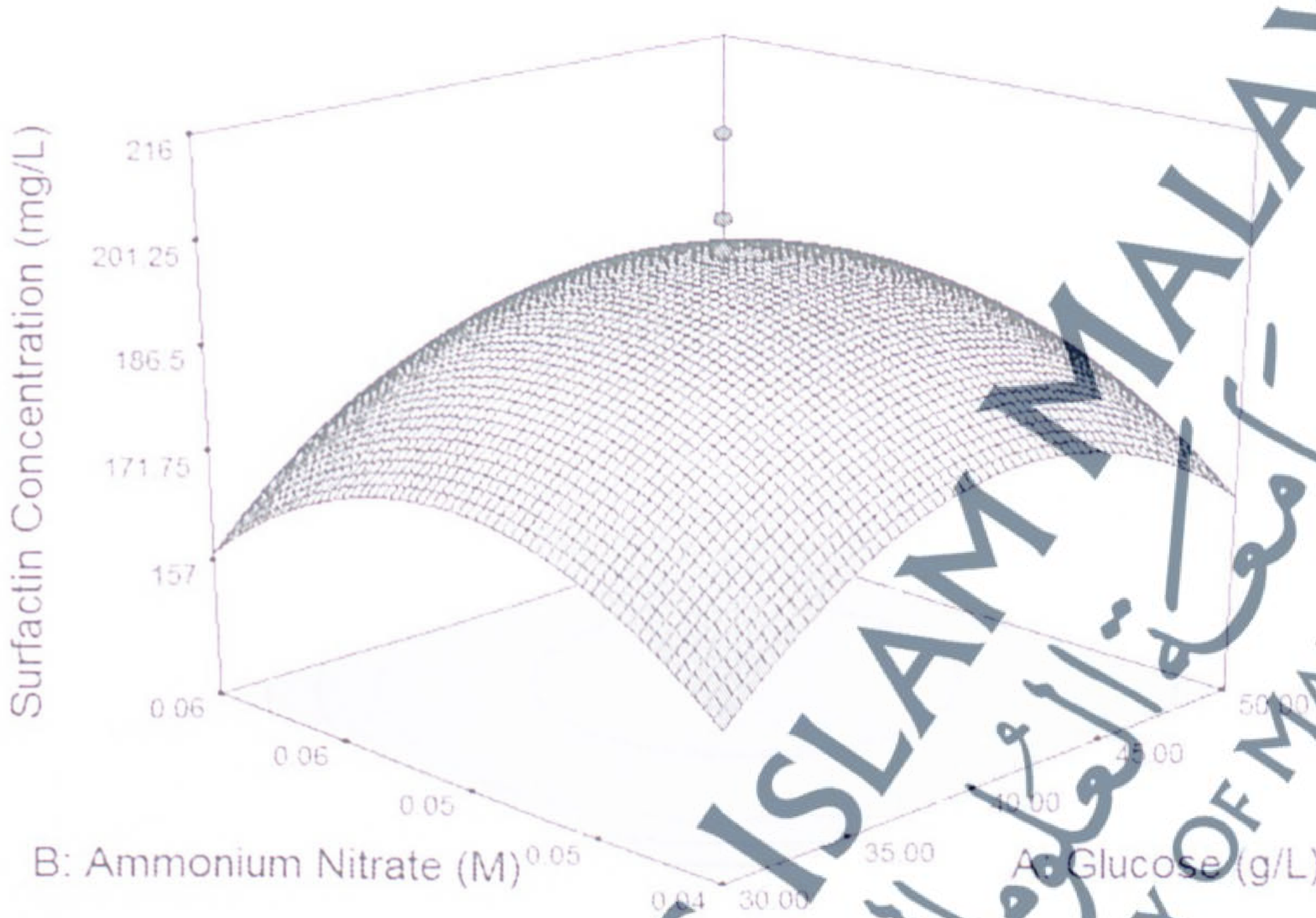
Figures 11 (a) and (b) illustrate the effect of various glucose and manganese sulfate concentrations on the production of surfactin and biomass respectively. From the 3D surface plots, the production of surfactin and biomass increased from 168.78 to 198.67 mg/L and 4.03 to 4.57 mg/L respectively by increasing the concentration of manganese sulfate to the optimum concentration of 1.65 and 1.82 mM respectively. However, when concentration of manganese sulfate reached 2.00 mM, surfactin and biomass production reduced to 189.81 and 4.28 mg/L respectively. The increment of surfactin production is

due to the function of Mn^{2+} in manganese sulfate that affects nitrogen utilization and K^+ uptake, as well as other biochemical functions (Wei and Chu, 2002). Hence, Mn^{2+} enhanced surfactin and biomass production up to the optimal idle concentration, which were about 198.67 and 4.57 mg/L respectively. In contrast, surfactin and biomass production decreased steadily to 189.81 and 4.28 mg/L when the concentration of manganese sulfate reached 2.00 mM. One of the possibilities is that this strain of *B. subtilis* has a defective manganese transport system when supplied by manganese sulfate in the range between 1.65 to 2.00 mM and thus inhibited surfactin production (Wei and Chu, 2002).

Figures 12 (a) and (b) depict the effect of various ferrous sulfate and manganese sulfate concentrations on the production of surfactin and biomass respectively. The 3D contour indicated that at low concentration of manganese sulfate and ferrous sulfate, surfactin and biomass production were low and yet increased gradually when the concentration of the both parameters in the medium increased. However, it also indicated that at very high concentrations of both parameters, low production of surfactin and biomass was observed. Meanwhile, Figures 13 (a) and (b) represent the interaction between various ammonium nitrate and manganese sulfate concentrations on the production of surfactin and biomass respectively. In contrast to the Figure 12 (b), however, the 3D contour plots in Figure 13 (b) showed a linear effect on biomass production when increasing the concentration of ammonium nitrate.

Figure 8: Response surface plots showing the effect of glucose (A) and ammonium nitrate (B) concentration with a fixed concentration of ferrous sulfate at 120 µM and manganese sulfate at 1.5 mM on the production of (a) surfactin and (b) biomass

a)



b)

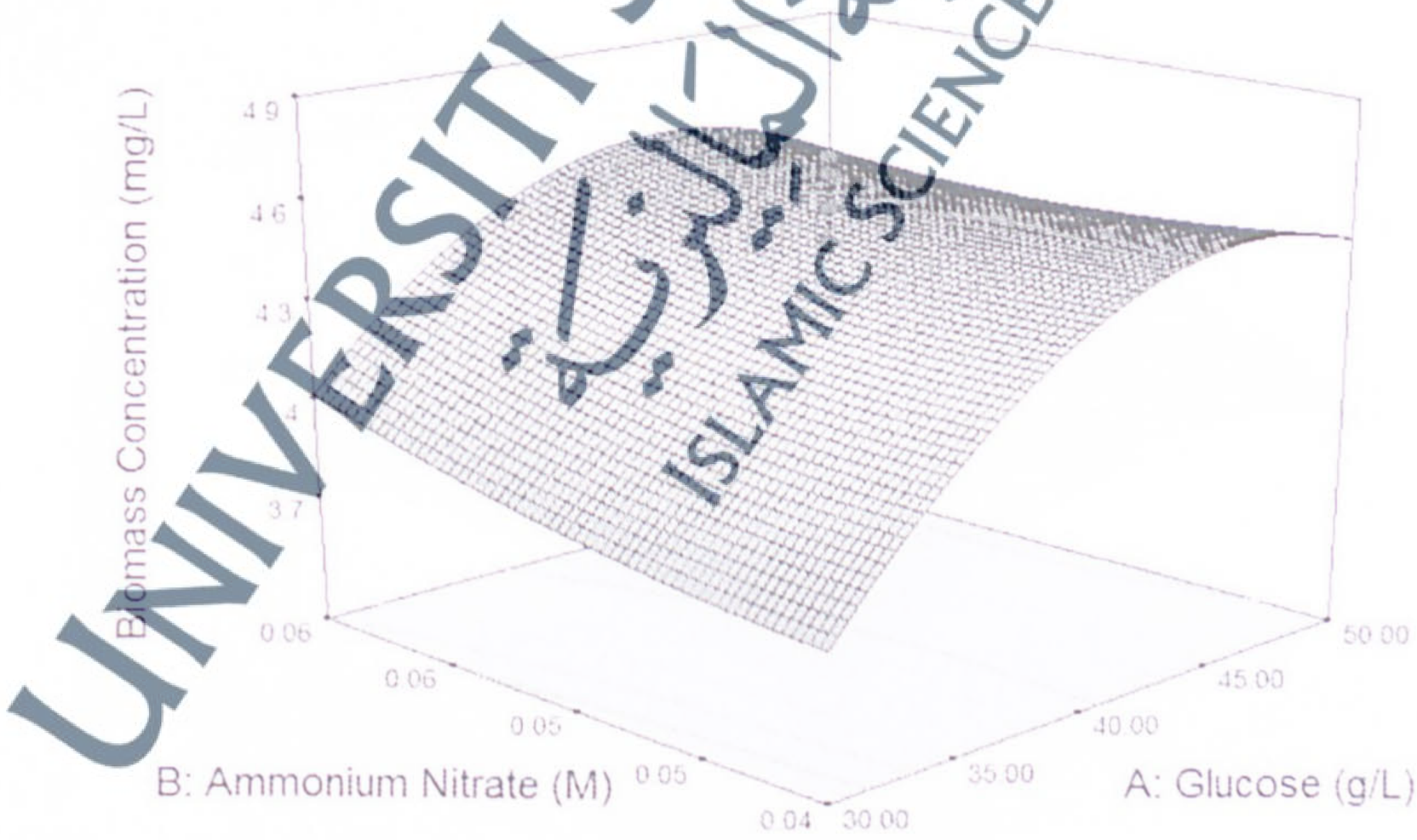
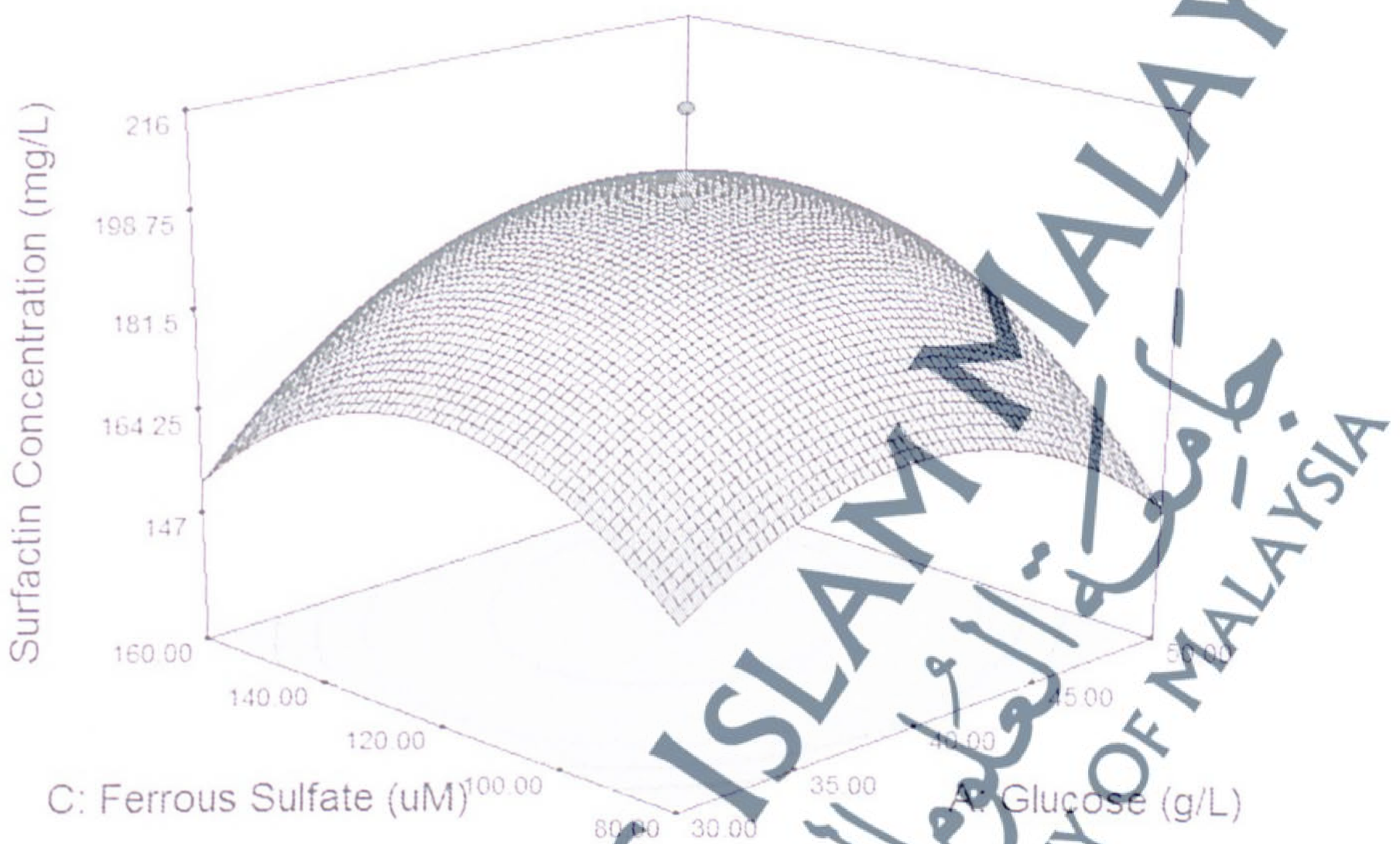


Figure 9: Response surface plots showing the effect of glucose (A) and ferrous sulfate (C) with a fixed concentration of ammonium nitrate at 0.05 M and manganese sulfate at 1.5 mM on the production of (a) surfactin and (b) biomass

a)



b)

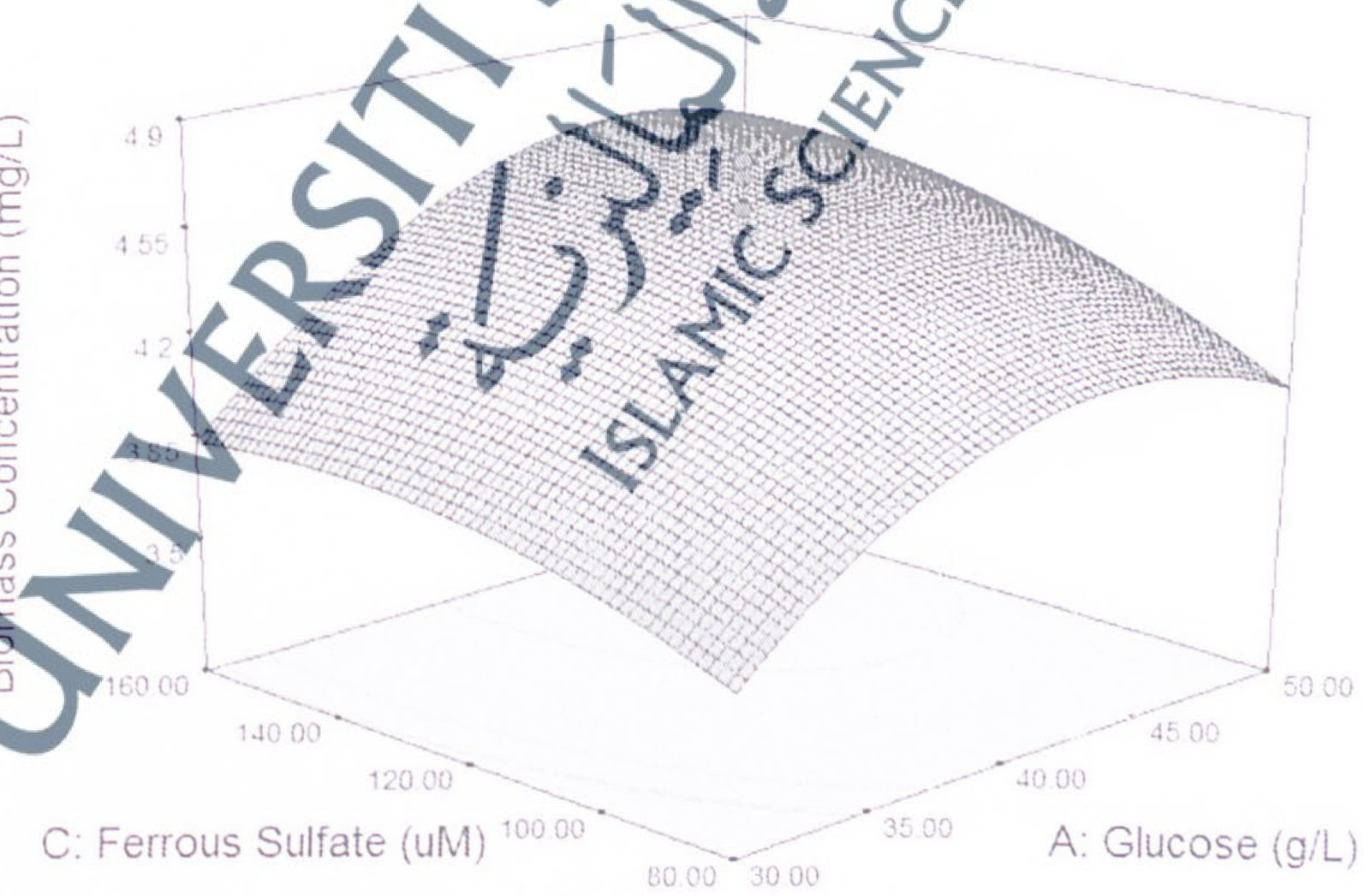
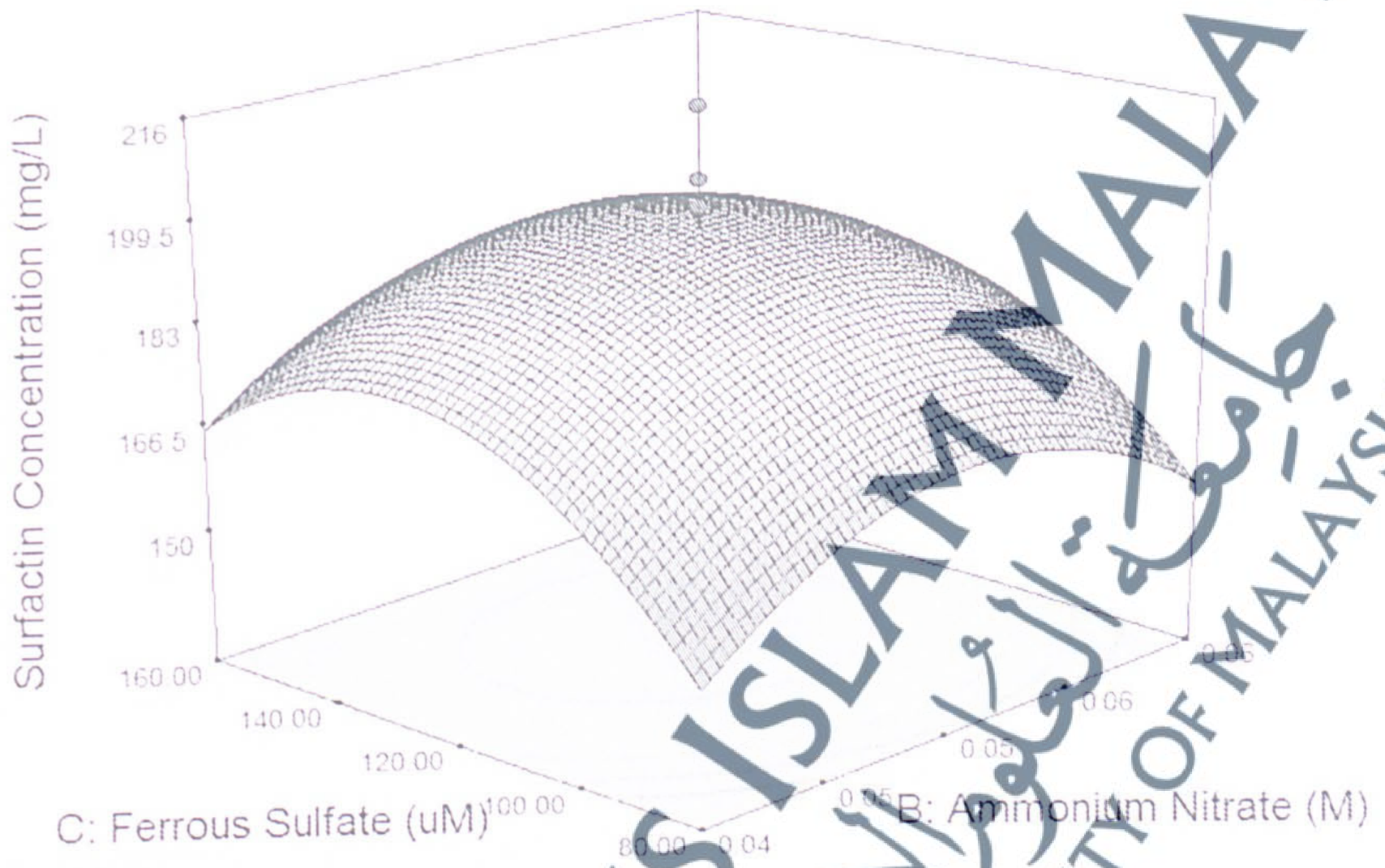


Figure 10: Response surface plot showing the effect of ammonium nitrate (B) and ferrous sulfate (C) concentration with a fixed concentration of glucose at 40 g/L and manganese sulfate at 1.5 mM on the production of (a) surfactin and (b) biomass

a)



b)

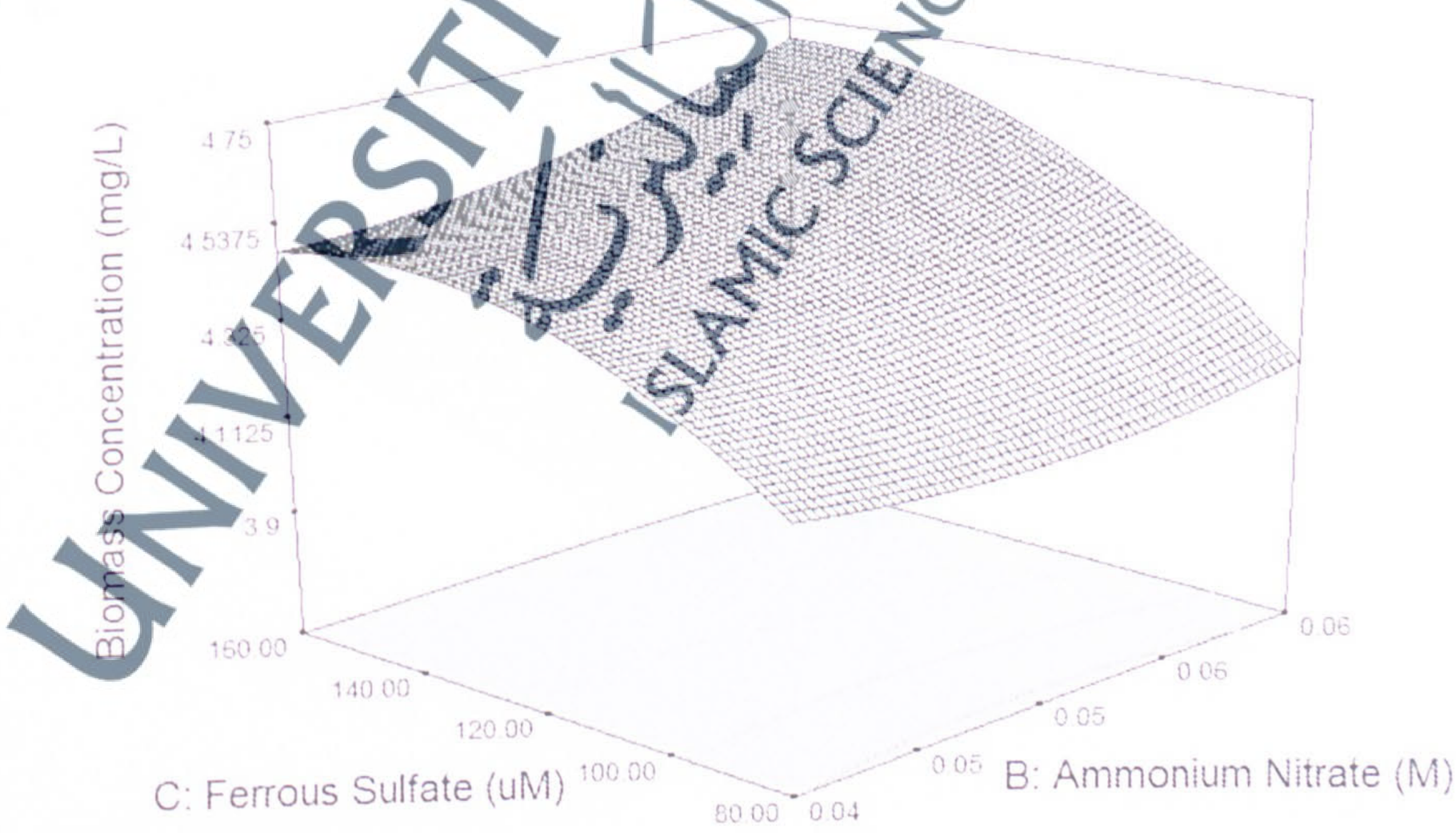
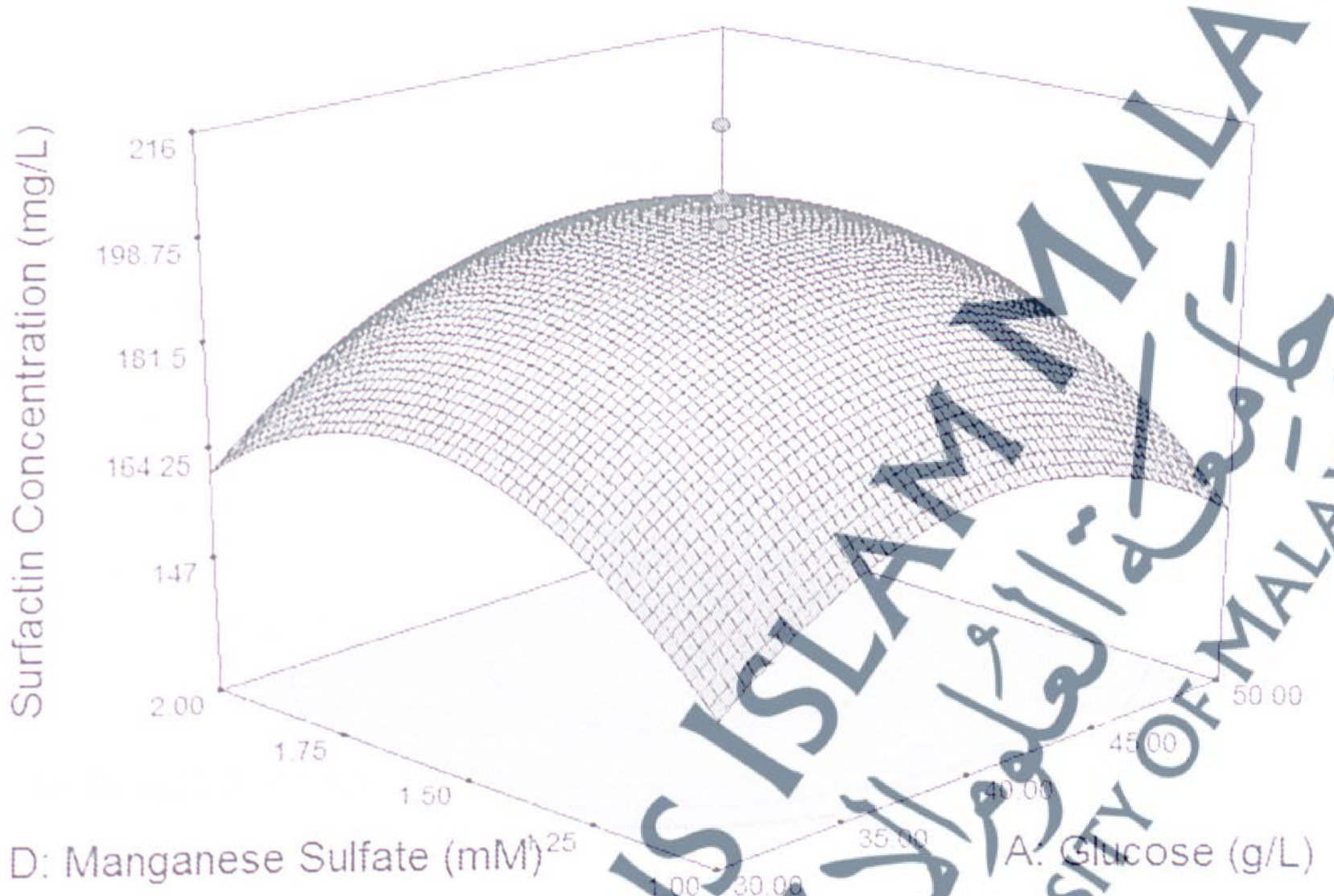


Figure 11: Response surface plot showing the effect of glucose (A) and manganese sulfate (D) with a fixed concentration of ammonium nitrate at 0.05 M and ferrous sulfate at 120 µM on the production of (a) surfactin and (b) biomass

a)



b)

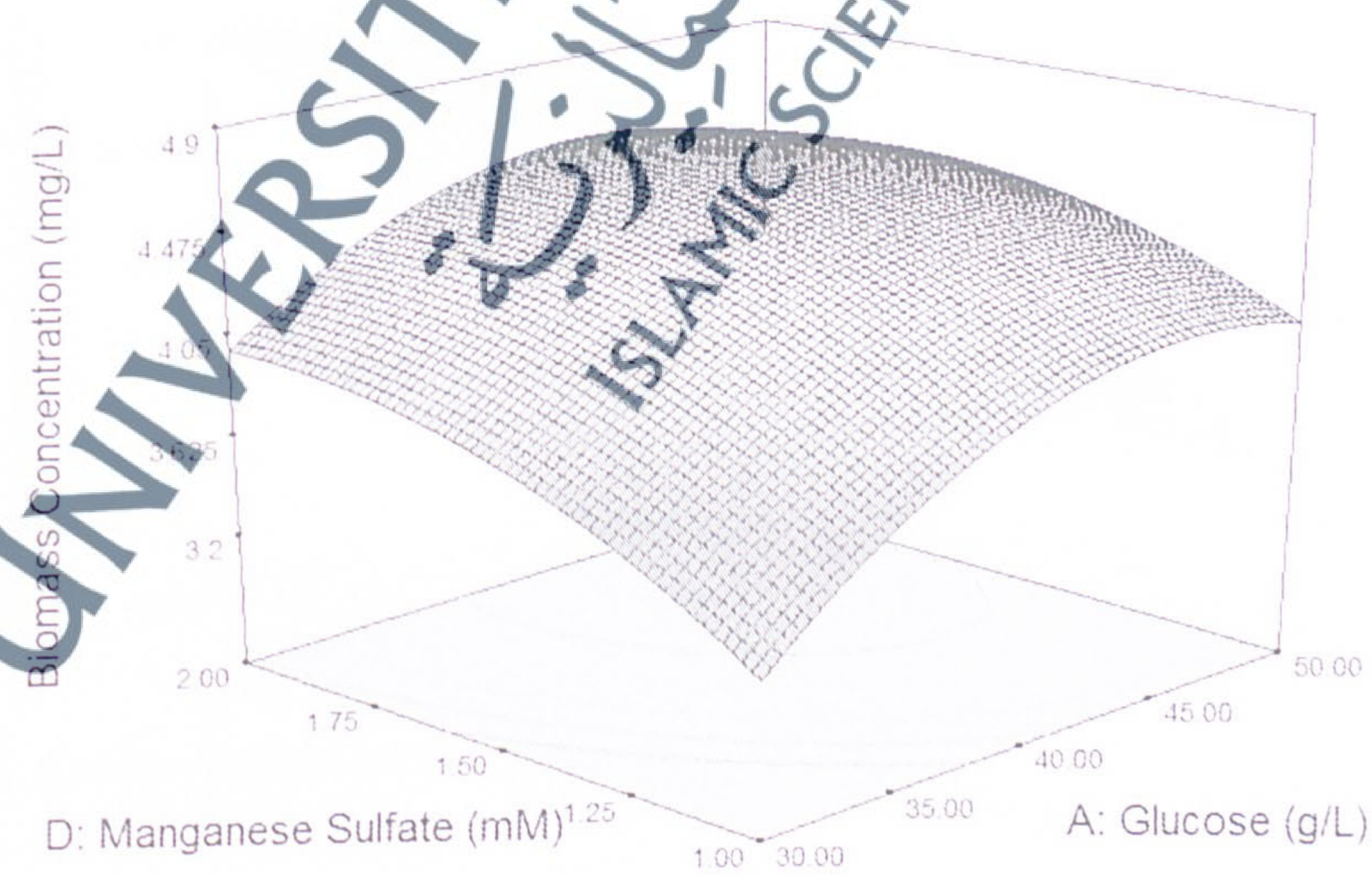
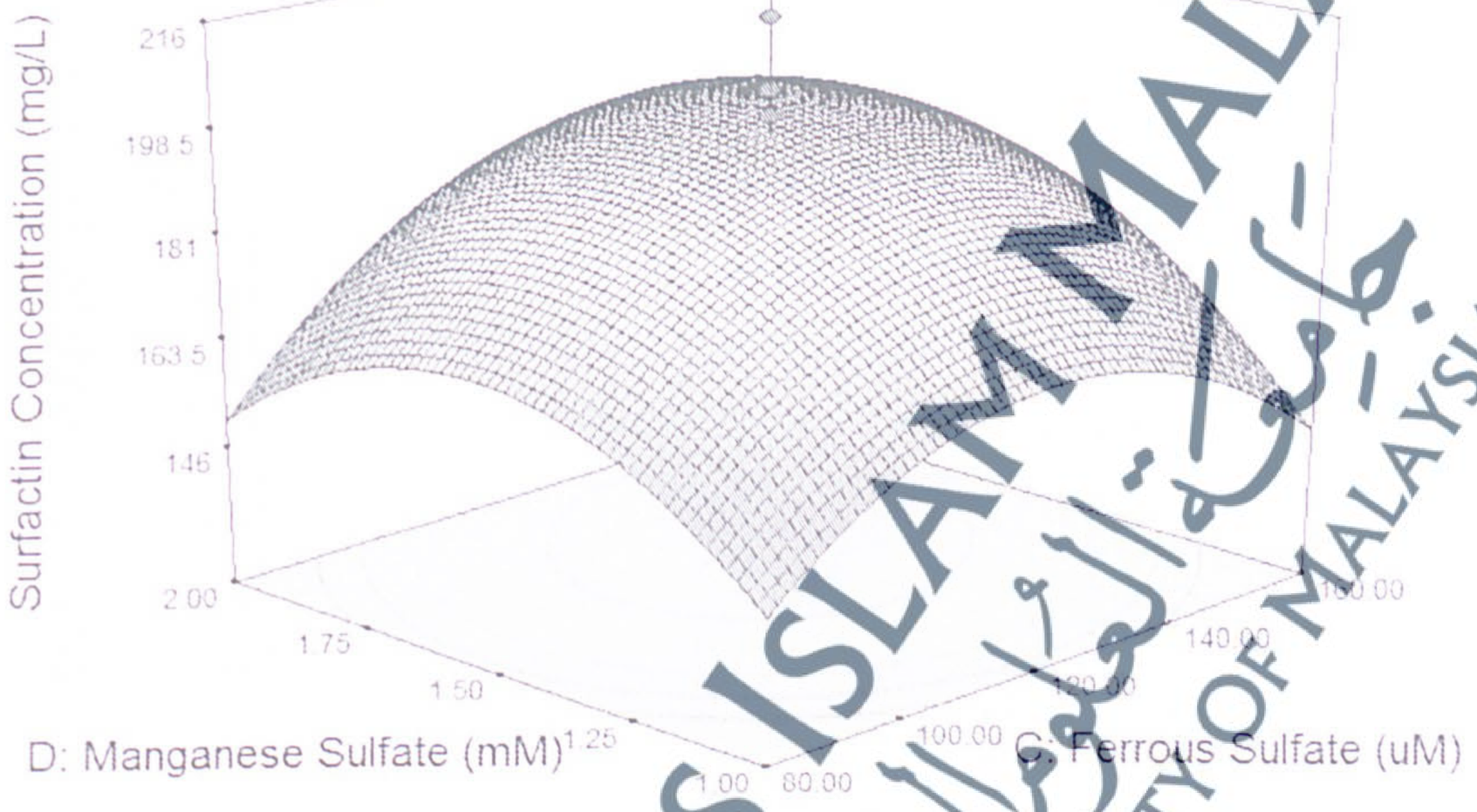


Figure 12: Response surface plot showing the effect of ferrous sulfate (C) and manganese sulfate (D) with a fixed concentration of glucose at 40 g/L and of ammonium nitrate at 0.05 M on the production of (a) surfactin and (b) biomass

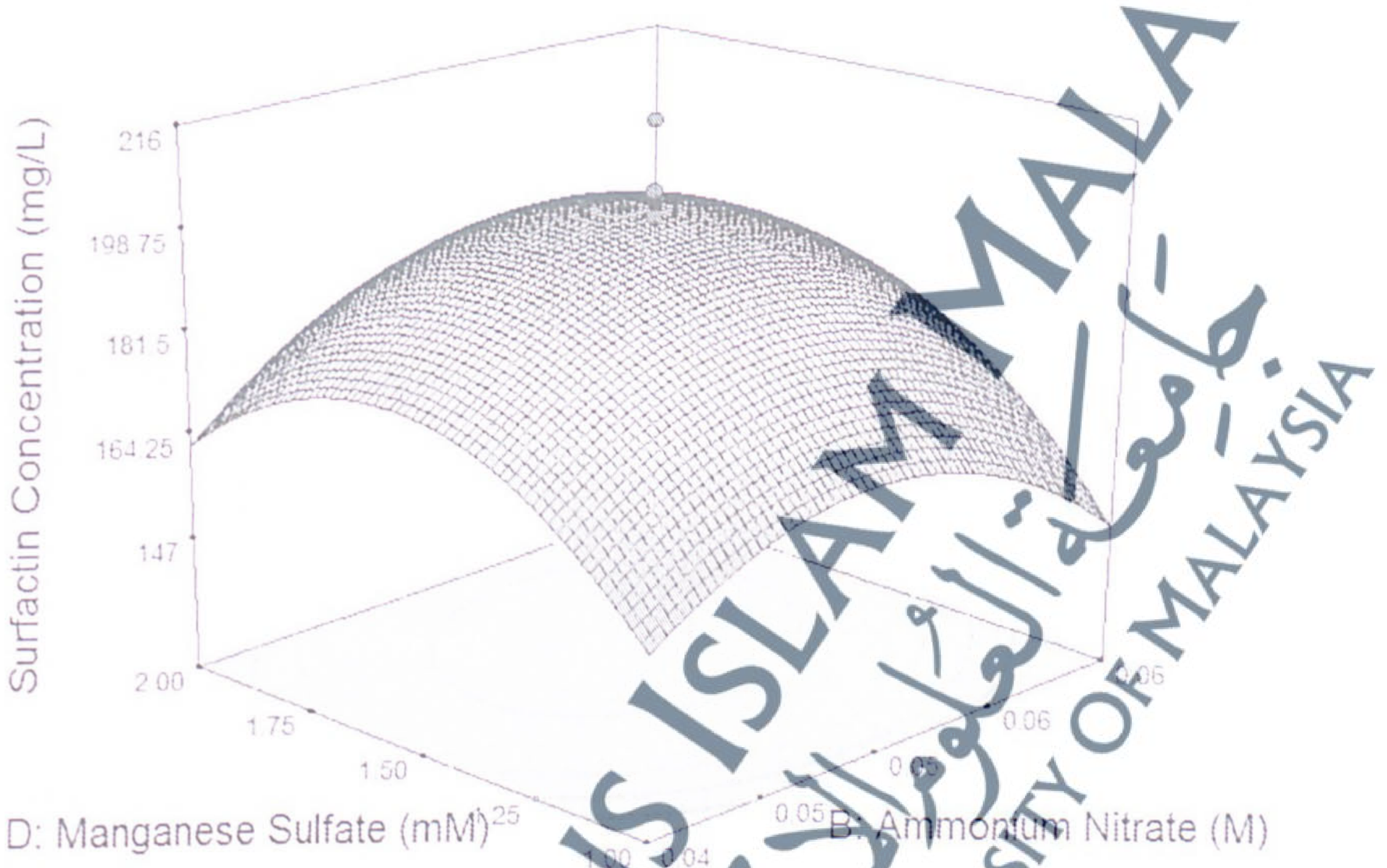
a)



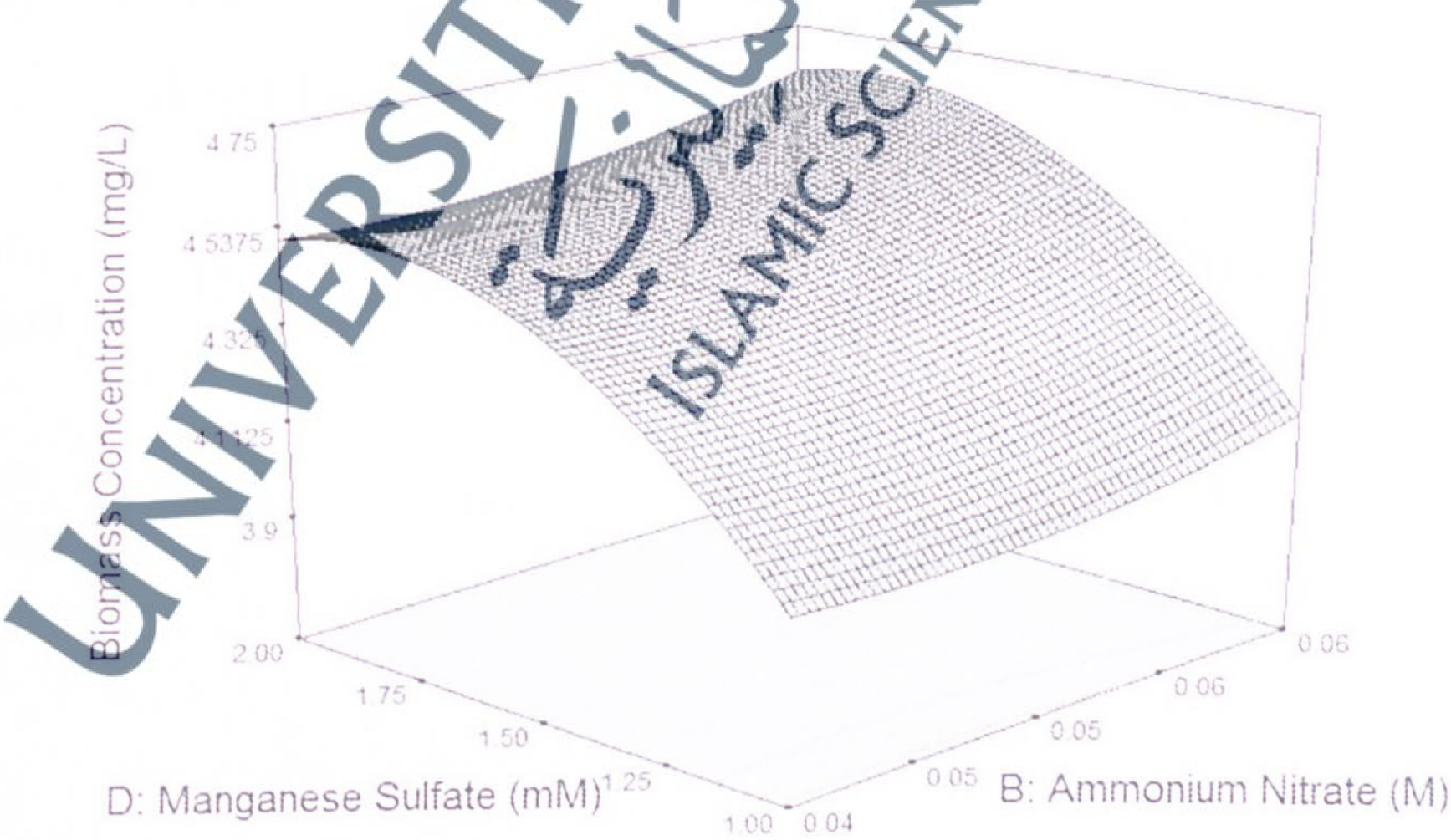
b)

Figure 13: Response surface plot showing the effect of ammonium nitrate (B) and manganese sulfate (D) with a fixed concentration of glucose at 40 g/L and ferrous sulfate at 120 µM on the production of (a) surfactin and (b) biomass

a)



b)



4.2.3 Verification of predictive model for surfactin and biomass production

The suitability of the model equation for predicting the optimum surfactin production values was tested by using the selected optimal conditions produced by the regression model. Validation experiments were carried out to verify the availability and accuracy of the model. Validation results in Table 12 show that the experimental value of surfactin production was very close to the predicted response and the predicted model fitted well with 98.34% of experimental results. Hence, the developed model was considered to be accurate and reliable for predicting the production of surfactin and biomass.

Table 12: Validation of model for surfactin and biomass production at optimum level of all parameters

Factors	Optimum condition				Experimental value (mg/L)	Predicted value (mg/L)
	A (g/L)	B (M)	C (µM)	D (mM)		
Surfactin production	42.6	0.051	134	1.64	190.8	200.6
Biomass production	42.6	0.06	160	1.97	4.81	4.92

4.3 OFAT optimization for fermentation conditions

For the second time, OFAT technique was employed to determine the effect and desirable ranges toward surfactin production. However, the parameters involved for this optimization experiment were fermentation time, inoculum volume and temperature. Subsequently, RSM optimization experiment was held with the same parameters.

4.3.1 Effect of fermentation time

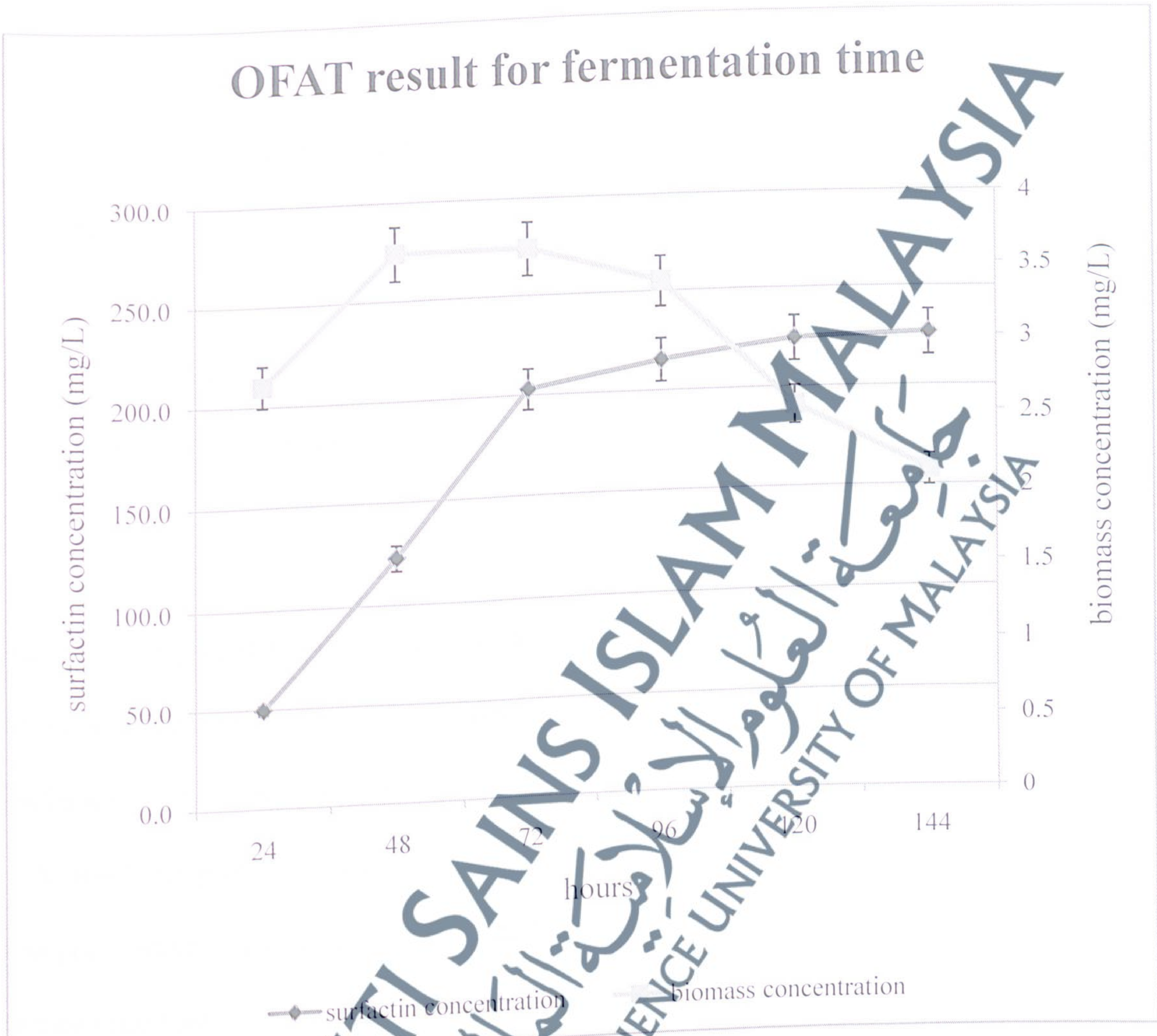
Figure 14 shows the effect of various fermentation times on the production of surfactin and biomass. In this optimization study, the sample was taken from the culture broth during the fermentation process at different time intervals of 24, 48, 72, 96 and 144 hours, while inoculum volume and temperature conditions were fixed at 5% and 30 °C respectively.

The result in Figure 14 indicates that with the increase of fermentation time from 24 to 72 h, surfactin production increased drastically from 49.6 to 203.0 mg/L. However, further increase of fermentation time from 72 to 144 h only showed a slight increase of surfactin production, which was from 203.0 to 226.5 mg/L. Since the production of surfactin between 72h and 144h was not huge in difference, 72h was chosen to be the optimal

fermentation time for subsequent OFAT experiment. In contrast, the fermentation time showed different effects on biomass production compared to surfactin production. From 24 to 48 h, yield of biomass increased from 2.8 to 3.65 mg/L and no increment was observed when fermentation continued up to 72 h. As fermentation proceed from 72 to 144 h, biomass production appeared to decline until reaching 2.1 mg/L.

The explanation for this surfactin and biomass production behavior was, at time interval between 24 and 48 h, *B. subtilis* microorganisms were in log or exponential phase of their cell growth activity, where they began to grow and divide in rapid state (Guo et al., 2010). In the course of the time, rapid multiplication of the microorganisms resulted in high accumulation of surfactin metabolites and biomass in the media. As fermentation continued from 48 to 72 h, *B. subtilis* in the media reached stationary phase, where reproduction rate was slow down and the cells underwent situation of cell division was equal to the number of cell death (Guo et al., 2010). Regardless, microorganisms managed to produce surfactin as usual since the growth rate was stabilized in this phase. Due to depletion of nutrient, *B. subtilis* lost their ability to reproduce and die after 72 h of fermentation. Thus, this death phase of fermentation reduced both biomass yielded in the media as well as surfactin production rate.

Figure 14: Effect of various fermentation times on the yield of surfactin and biomass produced by *B. subtilis* 3M



4.3.2 Effect of inoculum volume

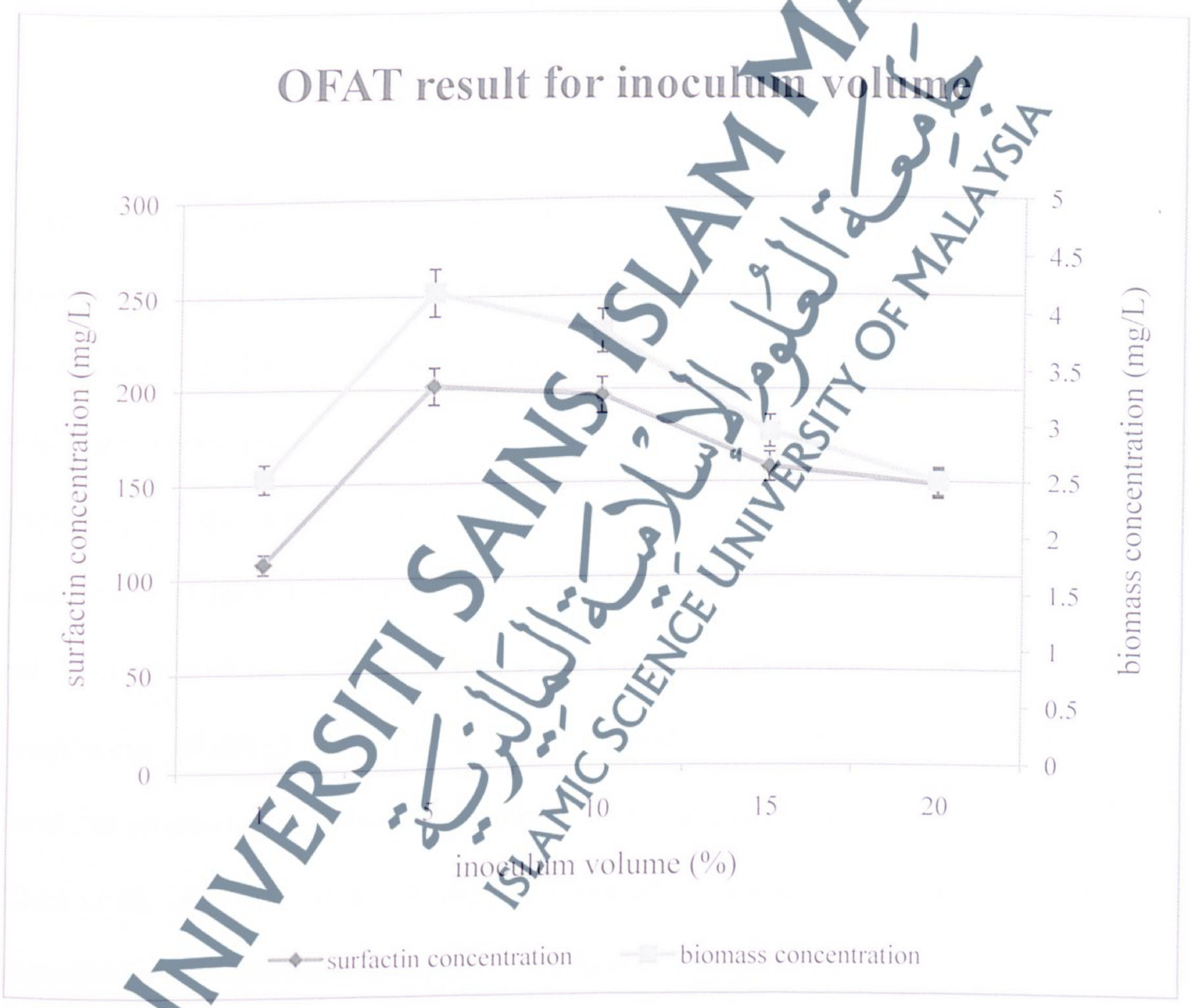
Inoculum volume is an important factor that could influence surfactin and biomass production. Although inoculum volume is rarely used as a factor in studies of microbial growth, there is evidence indicating that it may affect microbial growth. Hence, five different inoculum volumes ranging from 1 to 20 % were inoculated into the media to evaluate the effect inoculum volume on surfactin and biomass production after 72 h of fermentation at temperature of 30 °C

Figure 15 depicted that the surfactin and biomass production by *B. subtilis* 3M were influenced by inoculum volume. When inoculum volume increased from 1% to 5%, both surfactin and biomass productions were increased from 107.75 to 201.9 mg/L and 3.55 to 4.20 mg/L respectively. This means, the addition of 1% of inoculum volume into the Cooper's media was considered unsubstantial for microorganisms to provide suitable environment for high production of surfactin.

As the cell numbers in the inoculum increased up to 5 and 10%, high surfactin yield was achieved. The reason for this occurrence since the lag time phase decreased and thus fermentation process reached its log phase expeditiously. However, when the inoculum volume increased from 10 to 20%, both surfactin and biomass production were reduced from 196.6 to 148.05 mg/L and 5.2 to 3.5 mg/L respectively. This experiment indicates

that inoculum volume of 5% was sufficient to obtain good surfactin and biomass production. Hence, 5% was considered to be optimal inoculum volume and applied for the next temperature optimization experiment.

Figure 15: Effect of inoculum volume on production of surfactin and biomass produced by *B. subtilis* 3M

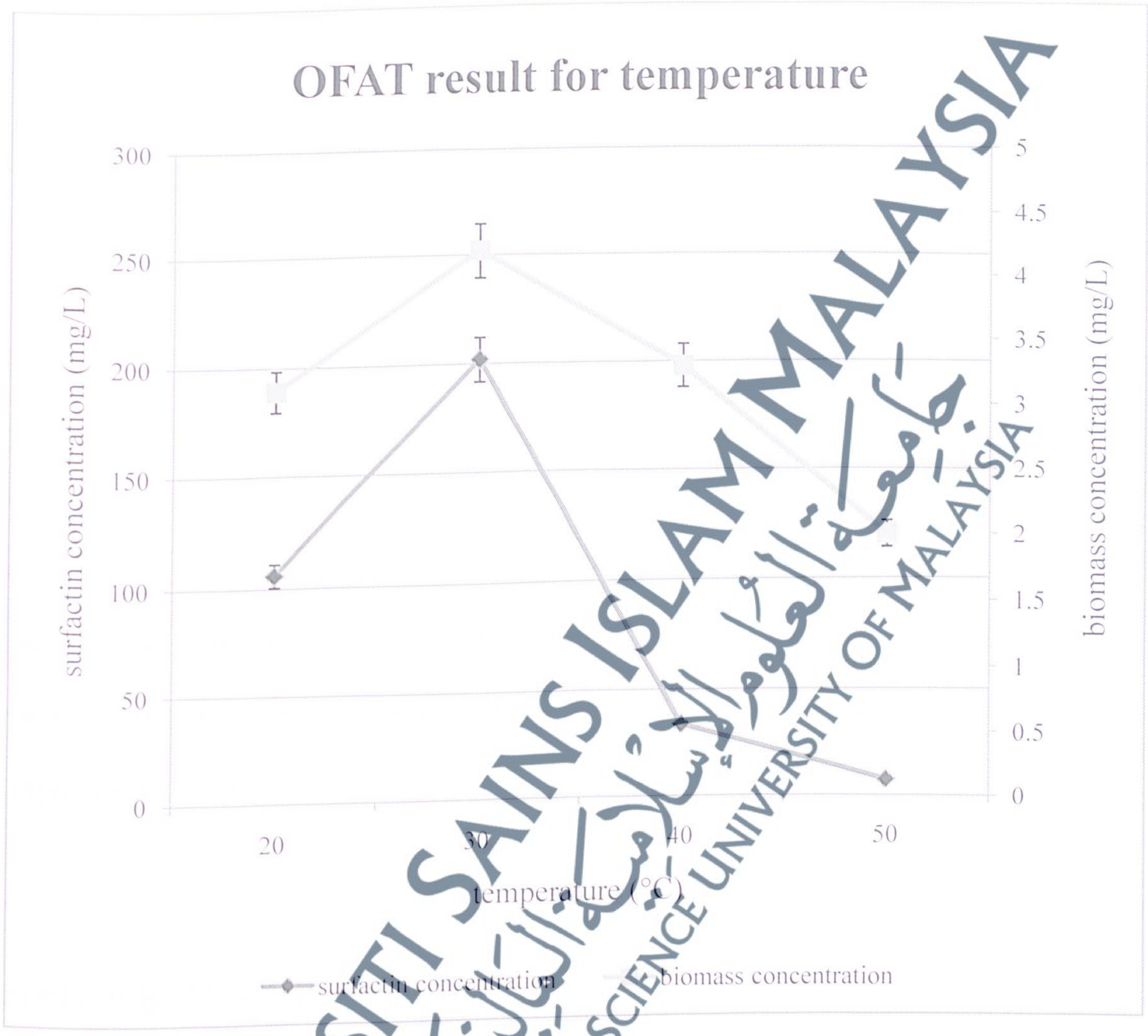


4.3.3 Effect of temperature

The temperature along fermentation process has a direct effect on the growth of the bacteria and thus affecting the surfactin production. In order to study the effect of different temperature on the surfactin production, a range of temperature varying from 20 to 50 °C was taken into study while the other fermentation conditions were fixed as follow: 72h of fermentation time and 5% of inoculum volume.

In this study, maximum surfactin and biomass production were observed at 201.9 and 4.2 mg/L respectively at 30 °C as represented in Figure 16. However, surfactin and biomass production started to decline from 201.9 to 7.5 mg/L and 4.2 to 2.0 mg/L respectively when the temperature reached 50 °C. The possible reason for this result maybe of suitability of the temperature required by the *B. subtilis* strain bacteria, which was unfavorable at high temperature of 50 °C (Mannan et al., 2005). Temperature of 30 °C was then considered a suitable condition for the bacteria since further increases of temperature inhibited growth of the bacteria and hence, decreased both biomass and surfactin production. This result is supported by various literatures (Cooper et al., 1981; Chen et al., 2008; Liu et al., 2009) that reported several *Bacillus* species bacteria were expected to yield maximum surfactin production at temperature of 30 °C.

Figure 16: Effect of temperature on production of surfactin and biomass produced by *B. subtilis* 3M



4.4 RSM optimization study for fermentation conditions

The results of the one-factor-at-a-time (OFAT) optimization of fermentation conditions from the previous study were used to determine the range of the parameters in this experiment. The variables that involved were fermentation time, inoculum volume and temperature, which showed significant factors on surfactin and biomass production.

Response surface methodology (RSM) is one of the statistical techniques to evaluate the relationship between the experimental variables within the design. By using central composite design (CCD), the optimum levels of the three selected variables that affected surfactin and biomass production were determined. The predicted mean and observed responses are presented in Table 13.

In this study, 20 experiments were augmented with five replications at the design centre point for estimating the purely experimental uncertainty variance as shown in Table 13, along with the predicted and experimental values of responses (biomass and surfactin concentrations). The statistical software package known as Design-Expert 7.1.6 was used for regression analysis of experimental data and plotting response surfaces. The following regression equation obtained after the analysis of variance (ANOVA) provided the levels of surfactin and biomass produced as a function of the values of fermentation time,

inoculum volume and temperature. The production of surfactin and biomass could be predicted by the following model equations:

$$\text{Surfactin concentration} = 179.44 + 20.65A + 8.81B - 48.82C - 7.81AB - 6.14AC - 12.91BC - 11.44A^2 - 27.85B^2 - 19.13C^2 \quad (3)$$

$$\text{Biomass concentration} = 3.67 - 0.53A + 0.14B - 0.28C + 0.36AB + 0.26AC + 0.094BC - 0.25A^2 - 0.010B^2 - 0.14C^2 \quad (4)$$

Where A, B and C are fermentation time (h), inoculum volume (%) and temperature (°C) respectively.

Table 13: Central composite design arrangement, responses and predicted values for surfactin and biomass yield

Run	A	B	C	Surfactin concentration		Biomass concentration	
				Experimental	Predicted	Experimental	Predicted
1	48	5	25	121.9	116.3	4.40	4.44
2	96	5	25	199.3	189.9	2.30	2.36
3	48	10	25	188.8	161.9	4.00	4.02
4	96	10	25	218.8	204.8	2.80	2.95
5	48	5	35	59.8	58.6	3.50	3.38
6	96	5	35	96.5	96.9	1.90	1.91
7	48	10	35	58.9	64.3	2.95	2.92
8	96	10	35	80.5	81.7	3.30	3.30
9	72	7.5	30	188.6	186.5	3.70	3.46
10	72	7.5	30	175.5	186.5	3.40	3.46
11	72	7.5	30	182.6	189.0	3.80	3.67
12	72	7.5	30	180.9	189.0	3.50	3.67
13	31.6	7.5	30	99.3	100.8	4.00	4.07
14	112.4	7.5	30	168.5	173.2	2.40	2.28
15	72	3.3	30	72.4	79.9	3.60	3.62
16	72	11.7	30	102.6	101.3	4.15	4.08
17	72	7.5	21.6	181.6	193.4	4.10	3.95
18	72	7.5	38.4	42.7	37.2	2.90	3.00
19	72	7.5	30	173.4	176.5	3.70	3.88
20	72	7.5	30	176.4	116.3	3.90	3.88

4.4.1 Analysis on production of surfactin and biomass

The analysis of variance (ANOVA) in design expert software was used for regression analysis for the obtained data to estimate the coefficient of the regression equation. The result of ANOVA analysis for the response surface quadratic model in Tables 14 and 15 indicates that the model equations derived by RSM could adequately be used to describe the surfactin and biomass production under the stated range of fermentation conditions, while the statistical significance of ANOVA analysis was evaluated using the *F*-value and *P*-value.

Based on ANOVA result in Tables 14 and 15, it is observed that the models for surfactin and biomass productions were highly significant, as it was evident from the *F*-test and a very low *P*-value. In the statistical analysis, *P*-value was used as a tool to check the significance of each of the coefficient. Low *P*-value ($p < 0.05$) indicates high significance of the corresponding coefficient and its effect on the surfactin and biomass production at 95% confidence level. In this experiment, the model *F*-values corresponding to surfactin and biomass were 59.76 and 29.37 respectively, and *P*-value of the models was less than 0.001.

The "lack of fit" test compared the residual error to the "pure error" from replicated design points. The *F*-values for "lack of fit" test against surfactin and biomass were 4.98 and 0.7 respectively. It implies that the lack of fit is not significant relative to the pure error, which was indicated toward the fitness of the model. Tables 14 and 15 also reveal that among the model terms in this study, A, C, A^2, B^2, C^2, and A, B, C, AB, AC, A^2, C^2 had very significant influence on surfactin and biomass production respectively.

On the other hand, the goodness of fit of the model can be checked by the R^2 (determination coefficient) and the adjusted R^2 (multiple correlation coefficient R). R^2 value provides a measurement of how much variability in the observed response values can be explained by the experimental factors and their interactions. For a good statistical model, the value of R^2 should be close to 1.0. Based on Tables 16 and 17, the coefficient of determination (R^2) for production of surfactin and biomass was found to be 0.9853 and 0.9706 respectively. Meanwhile, the adjusted R^2 and predicted R^2 values for the model were 0.9689 and 0.8333 respectively for surfactin production and for biomass production were 0.9376 and 0.7781 respectively. According to the result, the model was expected to predict the response more correctly in the present case.

In Tables 16 and 17 also, adequate precision is shown to compare the range of the predicted values at the design points to the average prediction error where ratios greater than 4.0 indicate adequate model discrimination. The adequate precision for surfactin and

biomass was 22.65 and 18.87 respectively for this model. This high value of adequate precision demonstrated that model is significant for the production of surfactin and biomass. On the other hand, the coefficient of variation (CV) measures the ratio between standard error of estimate with the mean value of the observed response as percentage, and the low value of the CV indicates a very high degree of precision and a good deal of reliability of the experimental values. In this experiment, CV was calculated to be 7.46% and 5.06% for surfactin and biomass respectively, indicating a greater reliability of the experiments performed. As a general rule, a model can be considered reasonably reproducible if its CV is not greater than 10%.

On the other hand, Figures 17 (a) and (b) show the parity plot of satisfactory correlation between the actual and predicted values, wherein, the points clustered around the diagonal line indicate the good fit of the model. The relationship between the actual and predicted concentration values for surfactin and biomass production was determined by the model Equations 4.3 and 4.4. From the graph, most points clearly were nearby the line adjustment, meaning that the experimentally determined values were nearly similar by the model.

Table 14: Analysis of variance (ANOVA) for all terms of model for optimization of surfactin production

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	57363.22	9	6373.69	59.76	< 0.0001
A-fermentation time	5826.32	1	5826.32	54.63	< 0.0001
B-inoculum volume	1059.52	1	1059.52	9.93	0.0136
C-temperature	32547.05	1	32547.05	305.15	< 0.0001
AB	488.28	1	488.28	4.58	0.0648
AC	301.35	1	301.35	2.83	0.1313
BC	1333.86	1	1333.86	12.51	0.0077
A^2	1885.05	1	1885.05	17.67	0.003
B^2	11165.99	1	11165.99	104.69	< 0.0001
C^2	5270.44	1	5270.44	49.41	0.0001
Residual	853.28	8	106.66		
Lack of Fit	761.53	5	152.31	4.98	0.1084
Pure Error	91.75	3	30.58		
Cor Total	60014.09	19			

Table 15: Analysis of variance (ANOVA) for all terms of model for optimization of biomass production

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	7.91	9	0.8783	29.37	< 0.0001
A-fermentation time	3.84	1	3.8391	128.39	< 0.0001
B-inoculum volume	0.26	1	0.2574	8.61	0.0189
C-temperature	1.10	1	1.0956	36.64	0.0003
AB	1.02	1	1.0153	33.95	0.0004
AC	0.53	1	0.5253	17.57	0.003
BC	0.07	1	0.0703	2.35	0.1637
A^2	0.89	1	0.8907	29.79	0.0006
B^2	0.00	1	0.0015	0.05	0.8309
C^2	0.29	1	0.2930	9.80	0.014
Residual	0.24	8	0.0299		
Lack of Fit	0.13	5	0.0258	0.70	0.6591
Pure Error	0.11	3	0.0367		
Cor Total	8.7005	19			

Table 16: ANOVA for regression in the optimization of surfactin production

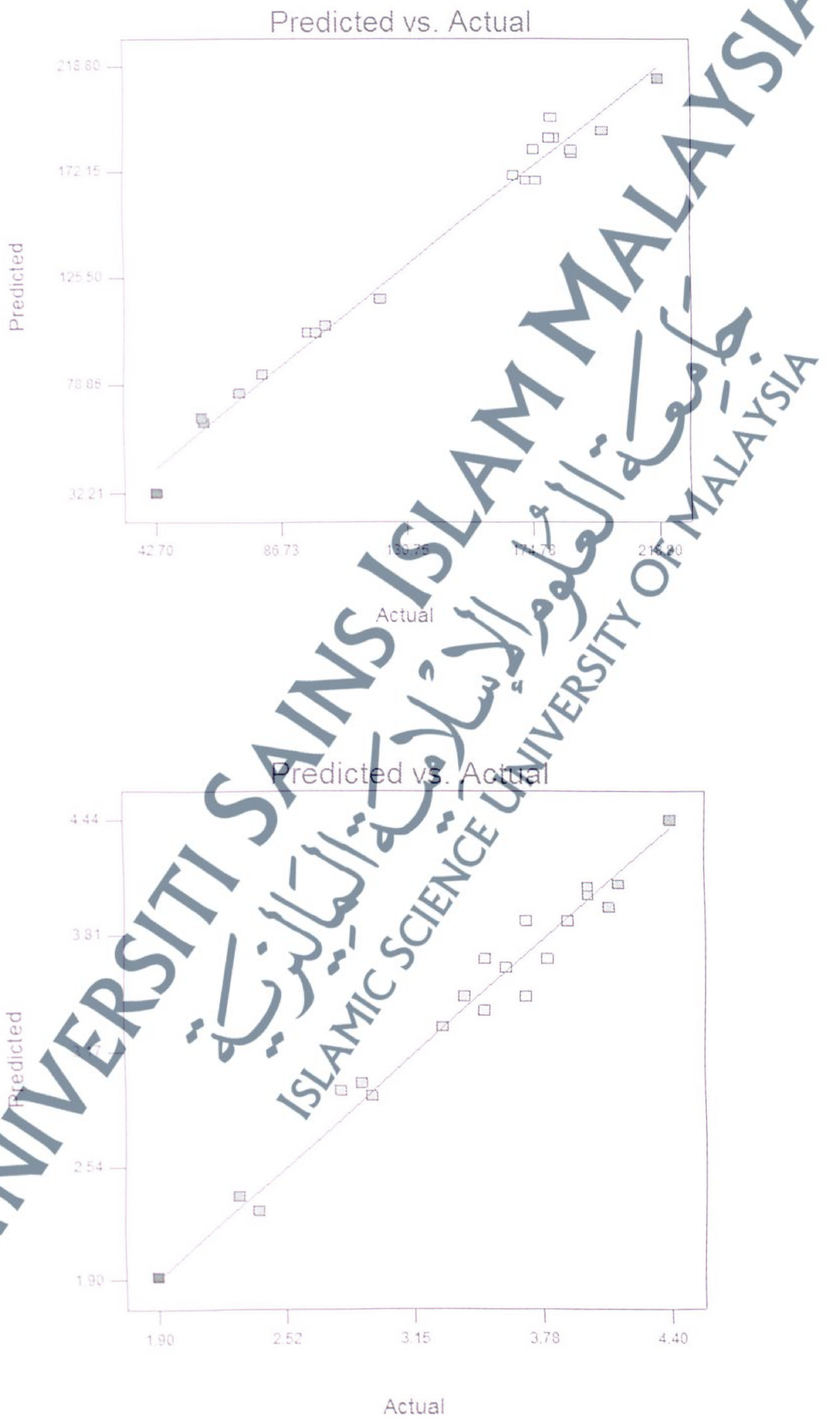
Terms	Value	Terms	Value
Std. Dev.	10.33	R-Squared	0.9853
Mean	138.45	Adj R-Squared	0.9689
C.V. %	7.46	Pred R-Squared	0.8333
PRESS	9706.58	Adeq Precision	22.651

Table 17: ANOVA for regression in the optimization of biomass production

Terms	Value	Terms	Value
Std. Dev.	0.17	R-Squared	0.9706
Mean	3.42	Adj R-Squared	0.9376
C.V. %	5.06	Pred R-Squared	0.7781
PRESS	1.81	Adeq Precision	18.865

Figures 17: A comparison between predicted and observed production of (a) surfactin and (b) biomass in mg/L

a)



b)

4.4.2 Combination effects of variables by response surface plots

The three dimensional response surface plots described by the regression model were drawn to illustrate the interactive effects of each independent variable on the response variables. In this experiment, three response surface graphs were obtained as developed by the quadratic equation model after considering all possible combinations.

The three dimensional graph were drawn by combinations of two parameters plotted at one time while another parameter was maintained at its respective zero levels (coded level: 0). Besides that, the statistical optimal values for the variables were obtained by moving along the major and minor axis of the contour, while the response yielded maximum surfactin and biomass production as described by respective dimensional response surface plots.

Therefore, the effects of the three factors as well as their combination effects on the surfactin and biomass production were drawn and shown in Figures 18, 19 and 20. Figures 18 (a) and (b) represent the three dimensional surface plots exhibiting the effect of fermentation time and inoculum volume in fixed average of temperature (30 °C) on the yield of surfactin and biomass respectively. Based on Figure 18 (a), surfactin concentration increased gradually from 148.3 to 178.0 mg/L when the fermentation time

increased from 48 until 77 hours. Then, from 77 until 96 hours, surfactin production showed only slightly increasing outcome, which was from 178.0 to 188.7 mg/L. According to the result, increment of fermentation time gradually enhanced surfactin production although at certain time, only slight increment of surfactin was observed. On the other hand, 3D surface plot for biomass production in Figure 18 (b) illustrates different contour nature in comparison with surfactin production. From the figure, biomass concentration decreased from 3.95 to 3.00 mg/L with increasing fermentation time from 48 to 96 hours. The possible explanation for this behavior is as fermentation continued from 48 to 96 h, microorganism in the media reached death phase, where number of cells in media started to reduce. Linear of surfactin production and declination of cell growth could be attributed to a number of factors such as depletion of glucose contents, metabolite accumulation as well as physicochemical changes in the process due to to changes in pH (Gurjar,and Sengupta 2015).

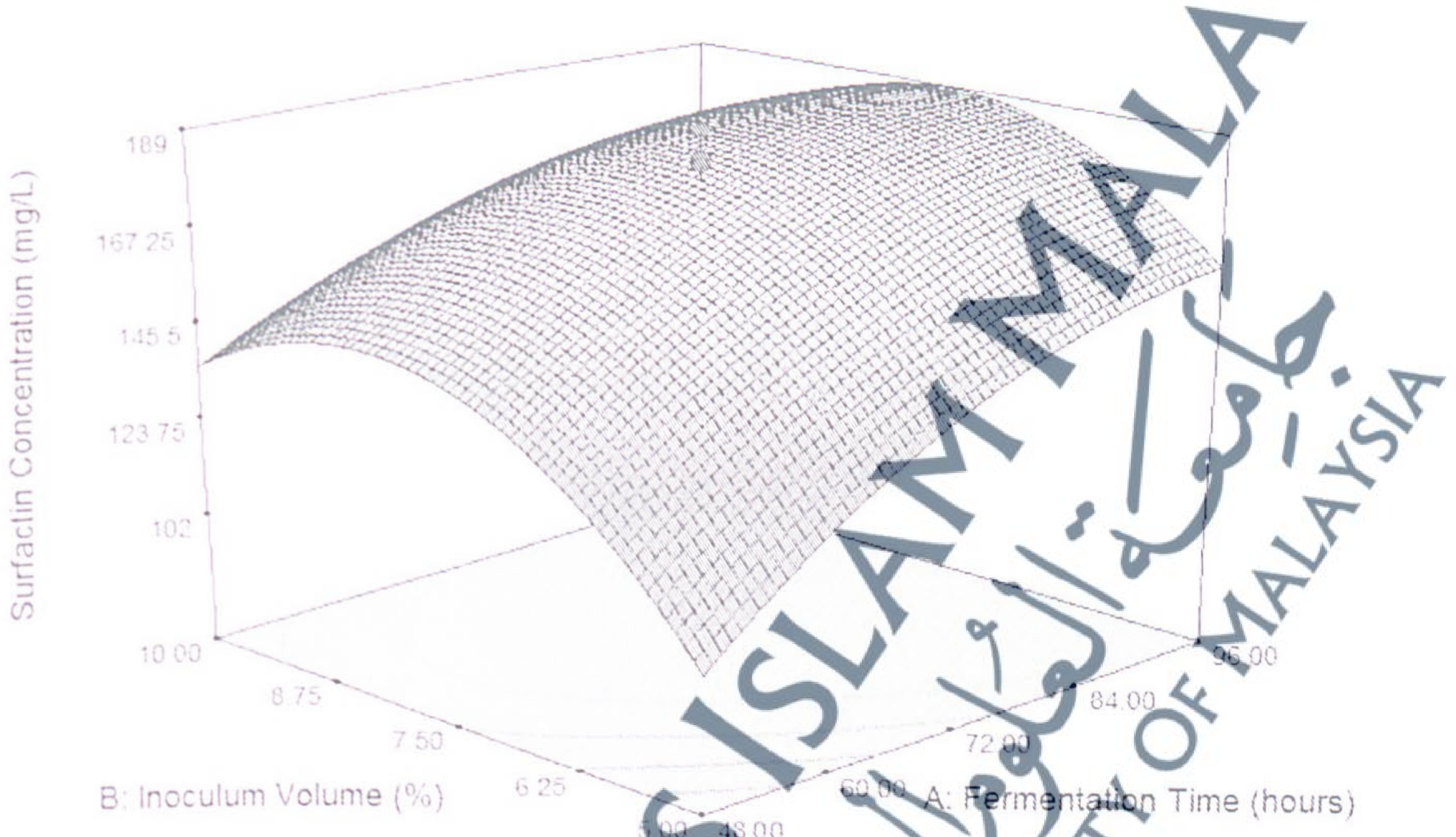
Figures 19 (a) and (b) show the effect of fermentation time and inoculum volume on surfactin and biomass production respectively. When the temperature increased from 25 to 35 °C, surfactin and biomass production decreased from 209.9 to 113.1 mg/L and 3.80 to 3.24 mg/L respectively. Based on the result, fermentation condition close to the temperature of 25 °C was considered suitable for the microorganism to produce surfactin. At that temperature, the growth of the cell activity was adversely increased, which had led to high surfactin and biomass yield. However, the temperature beyond 35 °C happened to decline both surfactin and biomass production. The explanation for this behavior rises

from warm environment condition during fermentation process, which is not convenient for the microorganism (Sen and Swaminathan, 1997). Furthermore, high temperature may affect cells by damaging and denaturing biomolecules such as proteins, DNA and RNA. There is also possibility of destabilization of cellular membrane structure hence, generating various adverse effects on membrane-associated processes (Woo et al., 2014).

The three dimensional contour plot in Figures 20 (a) and (b) depict the effect of inoculum volume and temperature on surfactin production. According to Figure 20 (a), inoculum volume shows quadratic effect on surfactin production and we can see that when inoculum volume increased from 5.0 to 7.9%, surfactin production increased from 142.8 to 179.0 mg/L. However, when the inoculum volume increased further, surfactin production began to decline. The decrease in the surfactin production after 7.9% was perhaps due to the increasing limitation of key nutrients, including oxygen, for cells at higher densities. On the other hand, Figure 20 (b) illustrates the effect of inoculum volume and temperature on biomass production. The contour plot showed inoculum volume slightly affected the biomass production when the temperature range was between 25 to 30 °C. However, when temperature increased more than 30 °C, inoculum volume showed significant effect on biomass production.

Figures 18: Response surface plot showing the effect of fermentation time (A) and inoculum volume (B) on the production of a) surfactin and b) biomass with a fixed temperature of 30 °C

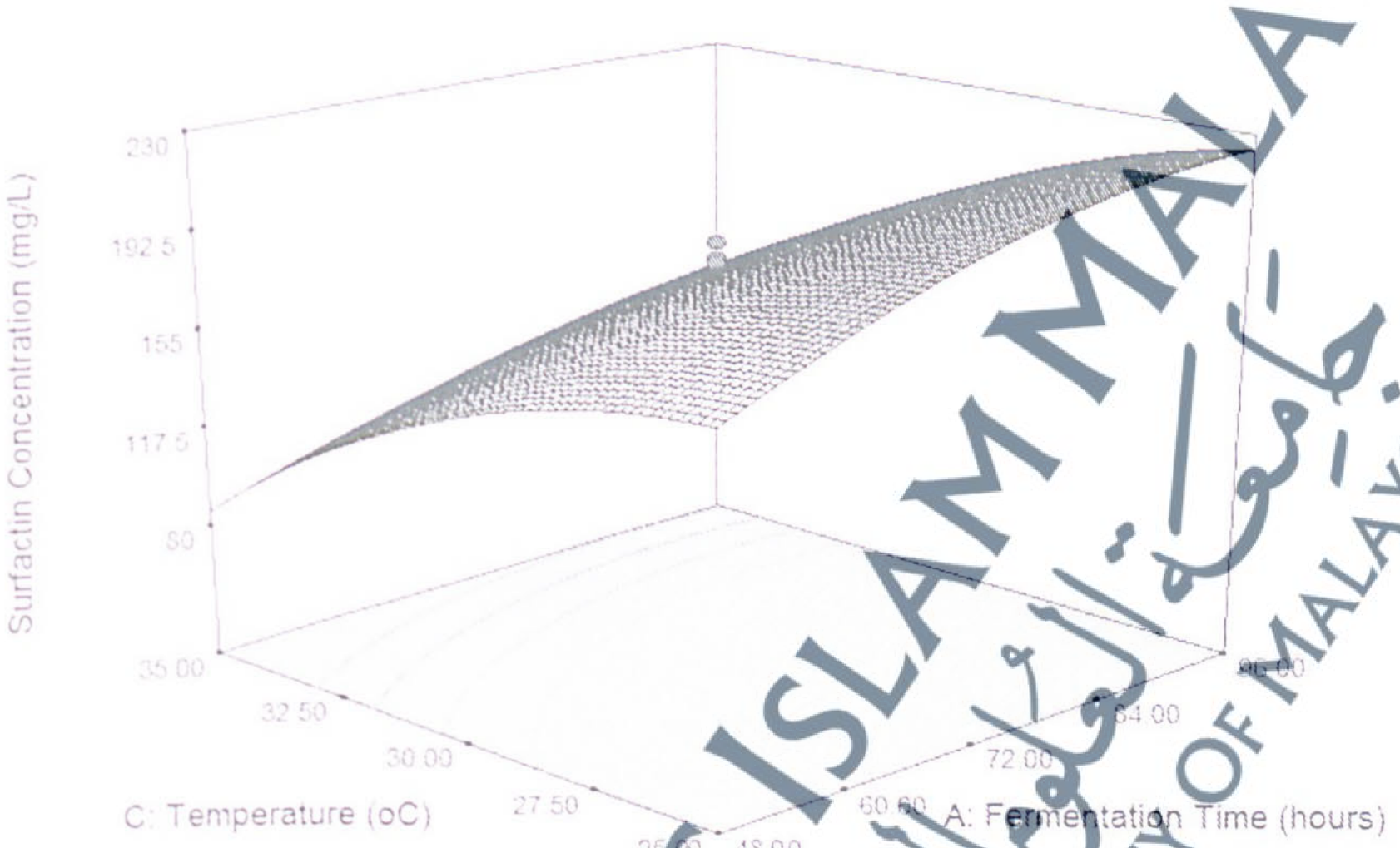
a)



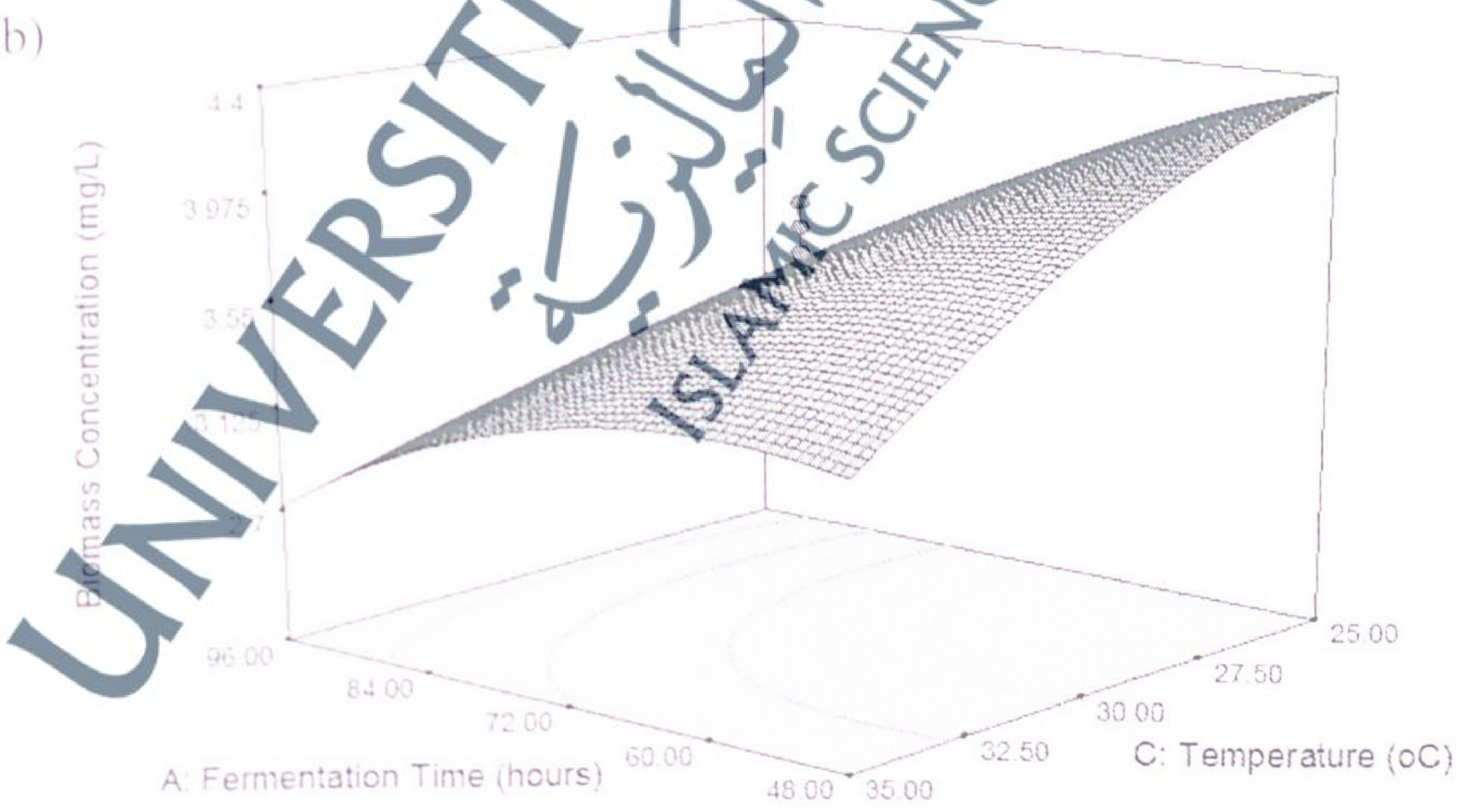
b)

Figures 19: Response surface plot showing the effect of fermentation time (A) and temperature (C) on the production of a) surfactin and b) biomass with a fixed inoculum volume of 5%

a)

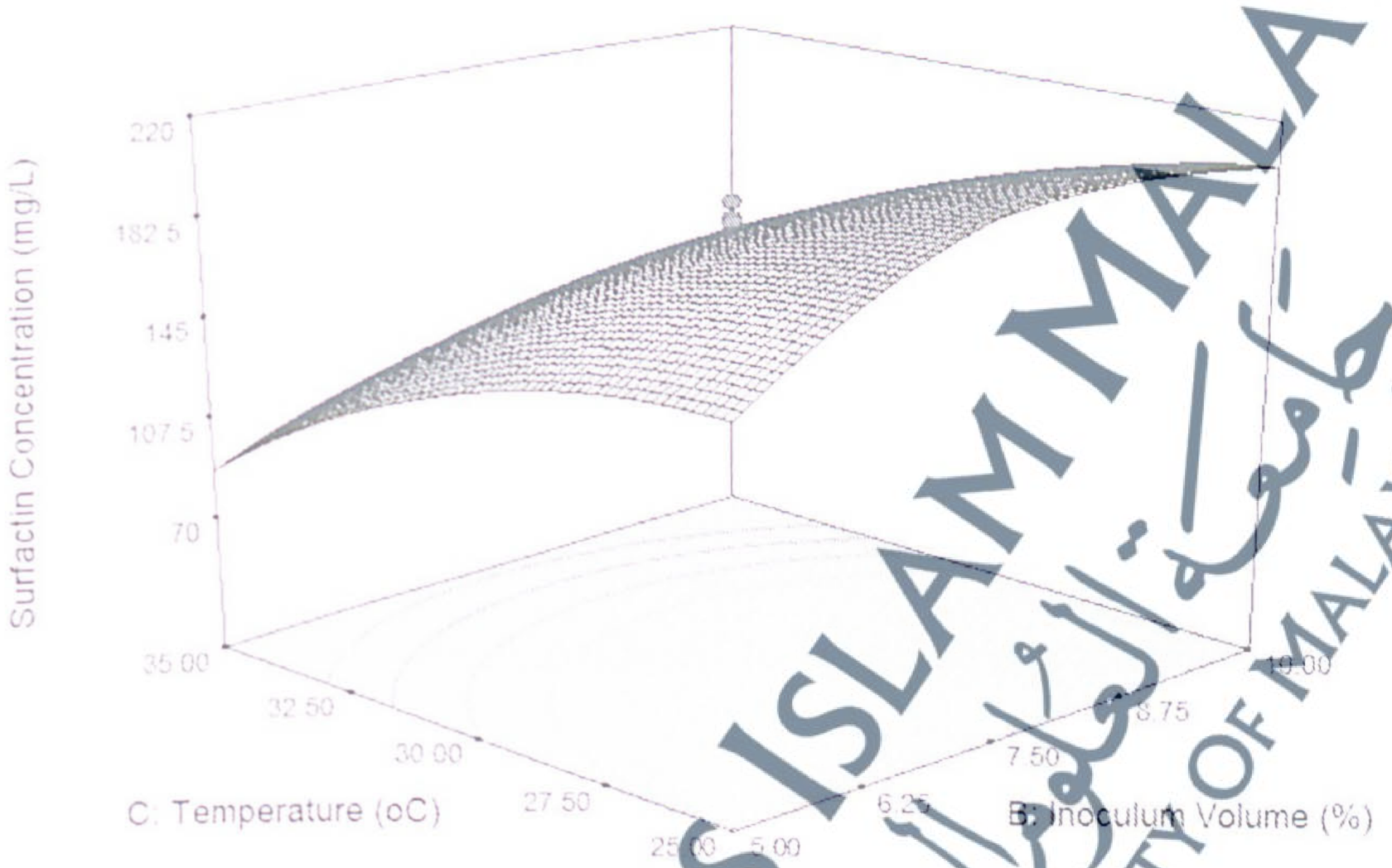


b)

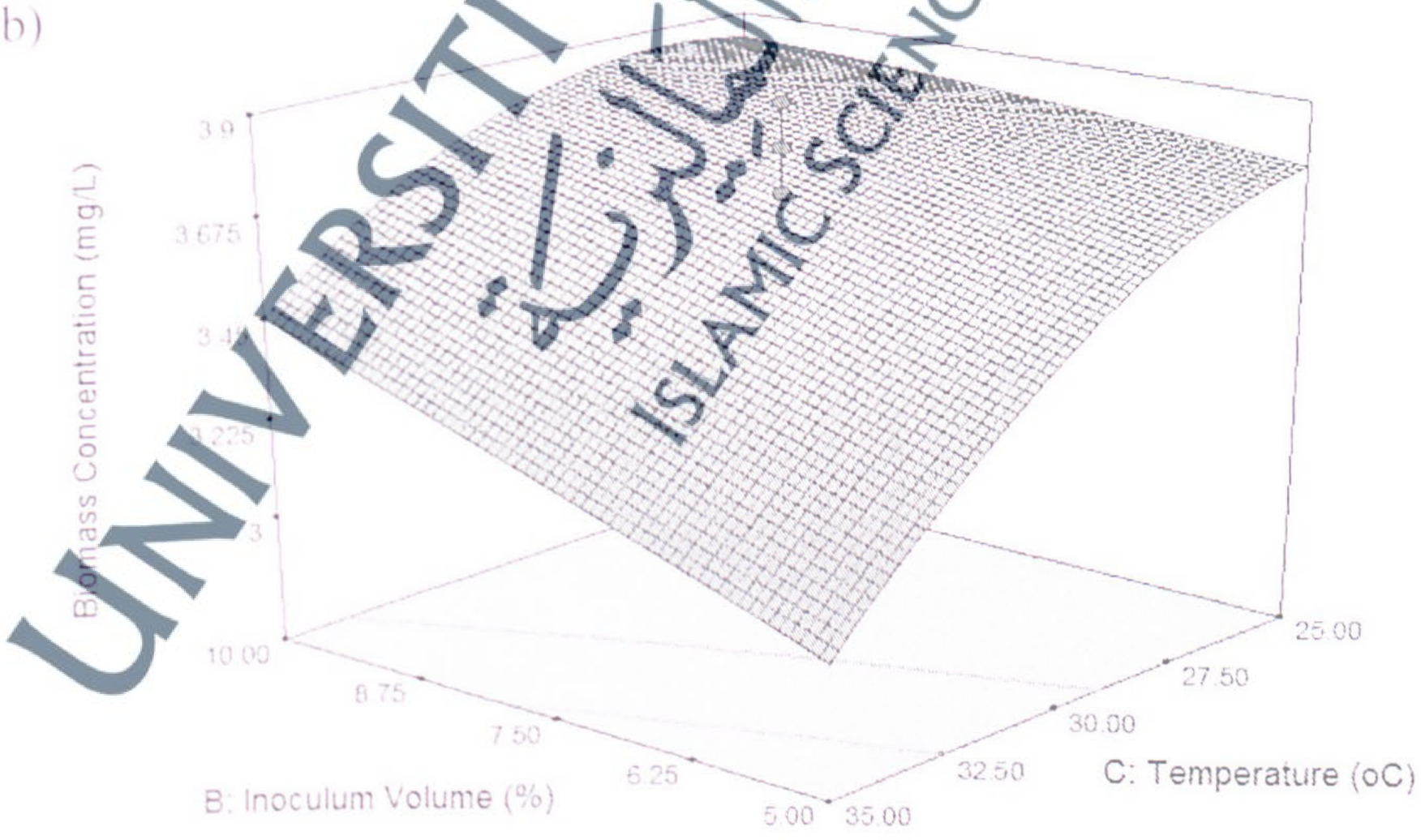


Figures 20: Response surface plot showing the effect of inoculum volume (B) and temperature (C) on the production of a) surfactin and b) biomass with a fixed fermentation time of 72h

a)



b)



4.4.3 Verification of predictive model for surfactin and biomass production

The suitability of the model equation for predicting the optimum production of surfactin and biomass values was tested using the optimization function of the Design Expert software. Validation results in Tables 18 showed that experimental value of surfactin and biomass production was very closer to the predicted response. Moreover, the comparison of experimental and predicted values revealed good correspondence between them, implying that the model derived from RSM can be used to adequately describe the relationship between the factors and response on production of surfactin and biomass. Hence, the model developed was considered to be accurate and reliable for predicting the production of surfactin and biomass.

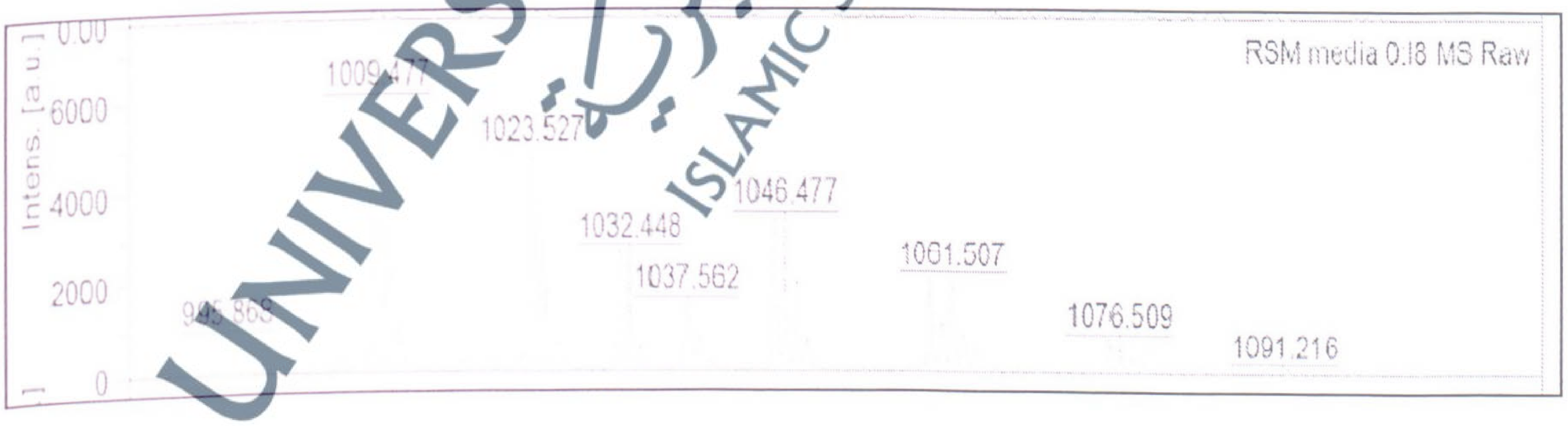
Table 18: Validation of model showing surfactin and biomass production at optimum level of all parameters

Factors	Optimum condition			Experimental value (mg/L)	Predicted value (mg/L)
	A (h)	B (%)	C (°C)		
Surfactin production	95	7.0	25	229.2	221.0
Biomass production	48	5.0	25	4.40	4.65

4.5 Mass spectrometry analysis

Although methods like ion exchange chromatography, thin layer chromatography, gel permeation chromatography and ultrafiltration have been used for the purification of lipopeptide biosurfactant, those techniques have a serious limitation as they do not separate individual isoforms present in the crude lipopeptide mixture (Sivapathasekaran et al., 2009). In this study, the crude surfactin yield by *B. subtilis* 3M and standard surfactin mixtures were introduced into C18 column of reversed-phase HPLC for purification and separation of the surfactin complex (see Appendix D). Subsequently, the molecule mixtures complex was investigated by MALDI-ToF mass spectrometry for mass determination. Figure 21 shows the MALDI mass spectra from fermentation broth obtained from HPLC purified fraction. Meanwhile, the distribution of the molecular ions of surfactin is summarized in Table 19.

Figure 21: Mass spectrum of surfactin from fermentation broth of *B. subtilis* 3M



The structure components of the surfactin complex which was analyzed by MALDI-ToF mass spectrometer were then further investigated and compared with reference compounds of surfactin isoforms as reported by Kowall et al (1998) (see Appendix E). Based on the report, surfactin of three different isoforms was detected in the form of added proton ($[M+H]^+$), ($[M+Na]^+$) and ($[M+K]^+$), as shown in Table 19. It is also apparent that the surfactin isoforms eluted according to their hydrophobic properties through observation in the mass range between *m/z* 1000 and 1100.

Table 19: MALDI-ToF mass spectrometric characterization of the surfactin produced by *B. subtilis* 3M

Mass peak (m/z)	Isoforms	Surfactin standard	Local isolate surfactin
994.2	Val-7, C-15 $[M+H]^+$	√	√
1008.7	Leu/Ile-7, C-13 $[M+H]^+$	√	√
1016.7	Val-7, C-13 $[M+Na]^+$	√	√
1022.7	Leu/Ile-7, C-14 $[M+H]^+$	√	√
1030.7	Leu/Ile-7, C-13 $[M+Na]^+$	√	√
1032.7	Val-7, C-13 $[M+K]^+$	√	√