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Effects of Mg^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} Ions on Lipid Accumulation by *Cunninghamella bainieri* 2A1

(Kesan Ion Mg^{2+}, Fe^{3+}, Mn^{2+} dan Cu^{2+} dalam Pengumpulan Lipid oleh
Cunninghamella bainieri 2A1)

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ABSTRACT

Cunninghamella bainieri *2A1 is an oleaginous fungus whose lipid accumulation profile is significantly influenced by metal ion concentrations in growth medium.* Mg^{2+}, Fe^{3+}, Mn^{2+} *and* Cu^{2+} *were found to be the important elements affecting lipid accumulation in this fungus. This study employs a statistical method (Response Surface Methodology – RSM) to study the combined effects of* Mg^{2+}, Fe^{3+}, Mn^{2+} *and* Cu^{2+} *on lipid accumulation of* C. baineri *2A1. Cultivation was carried out in 250 mL Erlenmeyer flasks containing 100 mL nitrogen limited medium at 30ºC and 250 rpm agitation for 120 h. A thirty-run central composite design experiment was employed to identify and optimize the significant factors. In addition to* Mg^{2+} *and* Fe^{3+} *which were shown to have significant effects on lipid accumulation, the interactions between* Mg^{2+} *and* Cu^{2+}*, as well as the effect of* Cu^{2+} *in quadratic terms were also found to have significant effect on the process* ($p<0.05$)*. The highest amount of lipid obtained in this study was 39% g/g biomass with optimal levels of* Mg^{2+}, Fe^{3+} *and* Cu^{2+} *at 5.00, 0.017 and 0.0005 g/L, respectively, while* Mn^{2+} *was omitted. A 32% increment in lipid yield was recorded, where the lipid content increased to 38%, compared to initial yield of 29% g/g biomass prior to optimization. In conclusion,* Mg^{2+} *and* Fe^{3+} *have significant positive effect on the lipid accumulation of this fungus, whereas* Mn^{2+} *and* Cu^{2+} *exert negative effects in combination.*

Keywords: Cunninghamella; *metal ions; response surface methodology; single cell oil*

ABSTRAK

Cunninghamella bainieri *2A1 merupakan kulat oleaginous dengan profil pengumpulan lipidnya dipengaruhi secara signifikan oleh kepekatan ion logam dalam medium pertumbuhan.* Mg^{2+}, Fe^{3+}, Mn^{2+} *dan* Cu^{2+} *adalah elemen penting yang mempengaruhi proses pengumpulan lipid dalam kulat ini. Kajian ini membabitkan penggunaan kaedah statistik (RSM) untuk mengkaji kesan kombinasi* Mg^{2+}, Fe^{3+}, Mn^{2+} *dan* Cu^{2+} *ke atas pengumpulan lipid oleh* C. baineri *2A1. Pengkulturan dilakukan dalam kelalang goncangan 250 mL yang mengandungi 100 mL medium terhad nitrogen pada suhu 30ºC dengan kadar goncangan 250 rpm. Set eksperimen yang terdiri daripada 30 larian reka bentuk gubahan memusat telah digunakan untuk mengenal pasti dan mengoptimumkan faktor yang signifikan. Di samping* Mg^{2+} *dan* Fe^{2+} *yang didapati mempunyai kesan yang signifikan ke atas proses pengumpulan lipid, interaksi antara* Mg^{2+} *dan* Cu^{2+} *serta kesan* Cu^{2+} *pada terma kuadratik juga mempunyai kesan yang signifikan dalam proses ini* ($p<0.05$)*. Peratus lipid maksimum yang tercapai adalah sebanyak 39% g/g biojisim dengan tahap optimum bagi* Mg^{2+}, Fe^{3+} *dan* Cu^{2+} *pada kepekatan 5.00, 0.017 dan 0.0005 g/L masing-masing, manakala* Mn^{2+} *tidak dimasukkan. Peratus peningkatan penghasilan lipid adalah sebanyak 32% dan ia meningkat kepada 38% berbanding dengan 29% g/g biojisim sebelum pengoptimuman. Kesimpulannya,* Mg^{2+} *dan* Fe^{3+} *mempunyai kesan positif yang signifikan ke atas proses pengumpulan lipid oleh kulat ini, manakala* Mn^{2+} *dan* Cu^{2+} *mempunyai kesan negatif secara kombinasi.*

Kata kunci: Cunninghamella*; ion logam; kaedah gerak balas permukaan*; *minyak sel tunggal*

INTRODUCTION

Oleaginous microbes are capable of producing more than 20% lipid per g biomass (Ratledge 1997) as a secondary metabolite which accumulates mainly during the stationary phase. Such lipid consists of unsaturated, monounsaturated (MUFA) and also polyunsaturated fatty acids (PUFA). Arachidonic acid (ARA), docosahexaenoic acid (DHA), eicosapentanoic acid (EPA) and gamma linolenic acid (GLA) are some examples of essential PUFAs which have high commercial value due to its nutritional benefits and pharmaceutical use (Dyal & Narine 2005). Microbial oil is found to be equal in quality with that from plant and animal origins. Thus, studies regarding the optimization of process conditions for commercial production of PUFAs from oleaginous microbes are of particular interest. This is crucial, since earlier attempts to commercialize single cell oil (SCO) were ceased due to uneconomic production costs (Ratledge 2005). Efforts to resume industrial level production of edible microbial oils are worth doing since essential PUFAs cannot be obtained in bulk from any other sources (Ratledge 2004).

A preferred way to enhance lipid production in batch cultivation of oleaginous microbes is by growing them in nitrogen limited medium (Ratledge 1997). A subject of interest is the metal elements, such as Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. The concentrations of metal ions in the medium can be altered in order to acheive the desired amount of lipid and also to amend the percentage of its constituents. These metal ions are known to act as cofactors for the enzymes involved in lipogenesis (Jasper & Silver 1997, Pirt 1975). However, inhibition of growth would occur at concentrations beyond the optimal points of the metal ions. Thus, determining the concentrations of metal ions in the medium that enhance lipid accumulation are vital. Increased productivity squares the high production cost, leading to viable commercialization of SCO.

In this study, we employed a local isolate, *Cunninghamella bainieri* 2A1 that was found to be capable of producing more than 20% (g/g) biomass lipid containing between 10 and 15% GLA (Aidil et al. 2001). A study conducted by Farhila et al. (2008) showed that lipid accumulation process in this fungus responds well to Mg^{2+}, Fe^{3+} and Zn^{2+}. However, the conventional approach used in the study did not take into account of the effect of interactions between those ions. The overal productivity were improved with a fixed combination of ion concentrations, which might differ from the individual optimal points. Therefore, in this study, we investigate the levels of significance of four metal ions used in nitrogen limited medium (Kendrick & Ratledge 1992), namely Mg^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} in lipid accumulation process of *Cunninghamella* 2A1 using RSM. This method enables graphical visualization of the pattern of interaction between these factor which collectively affects the amount of lipid accumulated by the fungus.

MATERIALS AND METHODS

INOCULUM PREPARATION

Cunninghamella bainieri 2A1 was obtained from the School of Bioscience and Biotechnology, Universiti Kebangsaan Malaysia and maintained on potato dextrose agar (PDA) at 4ºC. Standard inoculum was prepared from 7-day-old cultures grown on PDA. Seed culture was prepared by transferring the spore suspension into a 500 mL conical flask containing 200 mL of nitrogen limited medium (Kendrick & Ratledge 1992) to a final concentration of 10^5 spores/mL. The inoculum was incubated at 30ºC with 250 rpm agitation for 48 h.

CULTURE MEDIUM AND CULTURE CONDITION

Constituents of the seed culture medium are as follows (g/L): ammonium tartarate 1.0; KH_2PO_4 7.0; Na_2HPO_4 2.0; $MgSO_4.7H_2O$ 1.5; yeast extract 1.5; $CaCl_2$ 0.1; $Co(NO_3).6H_2O$ 0.0001; $FeCl_3.6H_2O$ 0.008; $ZnSO_4.7H_2O$ 0.0001; $CuSO_4.5H_2O$ 0.0001; $MnSO_4.5H_2O$ 0.0001; glucose 30.0 (added to the basal medium after being sterilized separately). The production medium contained all the components and composition as stated above, except for MgSO4.7H2O, $FeCl_3.6H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.5H_2O$, which concentrations were adjusted according to the design of experiment. Ten percent (v/v) of the seed culture was added into thirty 250 mL conical flasks containing 100 mL production medium. Range of the tested ion concentrations was set as shown in Table 1.

EXPERIMENTAL DETAILS

The cultures were incubated at 30ºC with 250 rpm agitation for 120 h. The results presented are the average value of triplicates.

TABLE 1. Range of concentration for the tested ions

Factor	Lower limit	Upper limit
A: Mg^{2+} (g/L)	0.250	5.000
B: Fe^{3+} (g/L)	0.004	0.030
C: Mn^{2+} (g/L)	0.000	0.010
D: Cu^{2+} (g/L)	0.000	0.001

Central composite design was used to study the system statistically (Box et al. 1978). A set of thirty runs with different combinations of ion concentrations was designed by the Design Expert 8.0.4.1 software as shown in Table 2. The outcome (percentage of lipid) was subjected to analysis of variance (ANOVA) using the same software in order to choose a correct model to explain the relationship and the interactions between the ion concentrations and the amount of lipid produced.

LIPID EXTRACTION

Five-day-old cultures in shake flasks (100 mL) were harvested by filtration through Whatman no.1 filter papers under low pressure. The mycelia were then rinsed twice with 100 mL distilled water and kept at -80ºC for 24 h before being freeze dried for another 24 h. The biomass of dried mycelia was determined prior to grinding it into powder. Lipid extraction was carried out using the method described by Folch et al. (1957).

RESULTS AND DISCUSSION

DATA ANALYSIS

The selection of the best model to explain the data was done based on the significance of sum of square (SS), Lack of Fit test and the R^2 value of each model. Tables 3, 4 and 5 show the SS, lack of fit test and R^2 values, respectively, for all four models involved.

TABLE 2. Experimental design by Design Expert software

Std	Run	A: Mg^{2+} (g/L)	B: Fe^{3+} (g/L)	C: Mn^{2+} (g/L)	D: Cu^{2+} (g/L)	Lipid yield (g/g biomass)	
						Actual	Predicted
19	1	2.6250	0.0040	0.0050	0.0005	28.65	27.32
20	2	2.6250	0.0300	0.0050	0.0005	33.69	30.68
24	3	2.6250	0.0170	0.0050	0.0010	22.83	19.11
26	4	2.6250	0.0170	0.0050	0.0005	24.41	28.21
22	5	2.6250	0.0170	0.0100	0.0005	33.40	30.94
8	6	5.0000	0.0300	0.0100	0.0000	35.03	34.03
14	7	5.0000	0.0040	0.0100	0.0010	22.82	23.11
28	8	2.6250	0.0170	0.0050	0.0005	22.75	28.21
23	9	2.6250	0.0170	0.0050	0.0000	21.82	21.21
30	10	2.6250	0.0170	0.0050	0.0005	27.49	28.21
15	11	0.2500	0.0300	0.0100	0.0010	24.94	28.23
3	12	0.2500	0.0300	0.0000	0.0000	22.06	22.42
21	13	2.6250	0.0170	0.0000	0.0005	32.05	30.18
29	14	2.6250	0.0170	0.0050	0.0005	25.61	28.21
9	15	0.2500	0.0040	0.0000	0.0010	20.77	22.43
17	16	0.2500	0.0170	0.0050	0.0005	31.37	29.73
10	17	5.0000	0.0040	0.0000	0.0010	28.74	30.91
18	18	5.0000	0.0170	0.0050	0.0005	37.81	35.12
13	19	0.2500	0.0040	0.0100	0.0010	24.03	21.36
27	20	2.6250	0.0170	0.0050	0.0005	25.82	28.21
1	21	0.2500	0.0040	0.0000	0.0000	20.48	19.81
5	22	0.2500	0.0040	0.0100	0.0000	24.14	26.23
2	23	5.0000	0.0040	0.0000	0.0000	34.23	31.59
25	24	2.6250	0.0170	0.0050	0.0005	30.16	28.21
12	25	5.0000	0.0300	0.0000	0.0010	33.56	32.12
7	26	0.2500	0.0300	0.0100	0.0000	33.48	31.74
16	27	5.0000	0.0300	0.0100	0.0010	26.11	27.21
11	28	0.2500	0.0300	0.0000	0.0010	27.08	26.41
4	29	5.0000	0.0300	0.0000	0.0000	28.34	31.44
6	30	5.0000	0.0040	0.0100	0.0000	30.18	31.29

TABLE 3. Sum of square (SS) values for statistical models

Source	SS	DF	$(\text{Mean})^2$	F value	P>F
Mean vs. total	23176.86	1	23176.86		
Linear vs. mean	203.74	4	50.94	2.7	0.0536
2FI vs. linear	130.25	6	21.71	1.21	0.3447
Quadratic vs. 2FI	179.90	4	44.97	4.18	0.0181
Cubic vs. quadratic	61.77	8	7.72	0.54	0.7955
Residue	99.76	7	14.25		
Total	23852.29	30	795.08		

$p<0.05$ is significant; DF is the degree of freedom

Based on the three numeric values shown in Tables 1-3, the quadratic model was chosen to explain the behaviour of the system, that is the connection between the variables (concentrations of ions) and the outcome (percentage of lipid produced). However, the cubic model has the highest R^2 value, which is 0.8523, compared to the quadratic model, which has the R^2 value of 0.7608 (Table 5). But, since the cubic model is insignificant in terms of SS value (Table 3) and is less insignificant in terms of lack of fit test (Table 4), the model is ignored. The percentage of variability explained by the quadratic model is 76.08% and the remaining 23.92% of the total variations is beyond the definition by this model.

Table 6 shows the regression coefficients, F values and P>F values for the factors studied and also the interaction among those factors. The P>F values for factors A (concentration of Mg^{2+}), B (concentration of Fe^{3+}), CD (interaction between the concentrations of Mn^{2+} and Cu^{2+}) and D^2 (interaction of Cu^{2+} concentration in quadratic terms) are significant, which implies that any slight changes

TABLE 4. Lack of Fit test

Source	SS	DF	$(\text{Mean})^2$	F value	P>F
Linear	438.90	20	21.94	3.35	0.0922
2FI	308.64	14	22.05	3.36	0.0937
Quadratic	128.75	10	12.87	1.96	0.2364
Cubic	66.97	2	33.49	5.11	0.0619
Pure error	32.79	5	6.56		

$p<0.05$ is significant; DF is the degree of freedom

TABLE 5. R^2 values

Source	Std. deviation	R^2	Modified R^2	Expected R^2	PRESS
Linear	4.34	0.3016	0.1899	0.0003	675.20
2FI	4.24	0.4945	0.2284	-0.1391	769.39
Quadratic	3.28	0.7608	0.5376	-0.0714	723.65
Cubic	3.78	0.8523	0.3881	-10.8538	8006.43

in these four factors will result in a marked change in the amount of lipid produced by this fungus.

Table 7 shows the regression coefficients, variance inflation factor (VIF) and standard error for the quadratic model. For all four factors and their interactions with other factors, VIF value equals to one, showing that the factors are independent and the model variance was not inflated by the lack of orthogonality in the design.

Given below is a polynomial equation that relates the yield (amount of lipid) and the concentrations of the ions:

$$\text{Amount of lipid, } Y = 28.21 + 2.69A + 1.68B + 0.38C - 1.05D - 0.69AB - 1.68AC - 0.83AD + 0.72BC + 0.34BD - 1.87CD + 4.22A^2 + 0.80B^2 + 2.35C^2 - 8.05D^2,$$

where Y is the percentage of lipid (g/g biomass); A is the concentration of Mg^{2+}(g/L); B is the concentration of Fe^{3+} (g/L); C is the concentration of Mn^{2+} (g/L) and D is the concentration of Cu^{2+} (g/L).

This result is supported by the findings of Farhila et al. (2008), where they found that Mg^{2+}, Fe^{3+} and Zn^{2+} ions have profound effect on lipid accumulation process in *Cunninghamella* 2A1, whereas Mn^{2+} and Cu^{2+} have no significant effect. However, the work was conducted using the conventional method, in contrast to the statistical method that was used in this study. Using this method, it was found that the interaction between the concentration of mangan and copper ions and the effect of copper in quadratic term have significant effect on the lipid accumulation process, even though the concentrations of the two ions has no significant effects individually. This indicates that copper affects the amount of lipid more

TABLE 6. Significance of the factors

Factor	Coefficient value	F value	P>F
Intercept	28.21		
A (Mg^{2+})	2.69	12.12	0.0033
B (Fe^{3+})	1.68	4.72	0.0462
C (Mn^{2+})	0.38	0.24	0.6313
D (Cu^{2+})	-1.05	1.84	0.1951
AB	-0.69	0.71	0.4123
AC	-1.68	4.21	0.0581
AD	-0.83	1.01	0.3302
BC	0.72	0.78	0.3920
BD	0.34	0.17	0.6839
CD	-1.87	5.21	0.0374
A^2	4.22	4.28	0.0564
B^2	0.80	0.15	0.7018
C^2	2.35	1.33	0.2669
D^2	-8.05	15.59	0.0013

$p<0.05$ is significant

TABLE 7. Regression analysis of quadratic model

Factor	Coefficient estimate	DF	Standard error	95% Cl low	95% Cl high	VIF
Intercept	28.21	1	1.02	26.03	30.38	
A-Mg^{2+}	2.69	1	0.77	1.04	4.34	1.00
B-Fe^{3+}	1.68	1	0.77	0.032	3.33	1.00
C-Mn^{2+}	0.38	1	0.77	-1.27	2.03	1.00
D-Cu^{2+}	-1.05	1	0.77	-2.70	0.60	1.00
A^2	4.22	1	2.04	-0.13	8.56	2.78
B^2	0.80	1	2.04	-3.55	5.14	2.78
C^2	2.35	1	2.04	-1.99	6.70	2.78
D^2	-8.05	1	2.04	-12.39	-3.70	2.78
AB	-0.69	1	0.82	-2.44	1.06	1.00
AC	-1.68	1	0.82	-3.43	0.066	1.00
AD	-0.83	1	0.82	-2.57	0.92	1.00
BC	0.72	1	0.82	-1.03	2.47	1.00
BD	0.34	1	0.82	-1.41	2.09	1.00
CD	-1.87	1	0.82	-3.62	-0.12	1.00

significantly at higher concentrations. Alterations in the range of Cu^{2+} (increased upper limit) incorporated in the medium would give a clearer picture about the individual effect of this ion.

SURFACE AND CONTOUR PLOT INTERPRETATION

The pattern of interaction between each pair of ions (at fixed concentrations of the remaining two ions) is presented graphically in the form of 3D surface plot and 2D contour plot as shown in Figures 1-6.

Except for the interaction between mangan and copper ions, all other pairs have no significant effect on the accumulation of lipid in *C. baineri* 2A1. The surface plot gives a very clear and interactive depiction on how the response (percentage of lipid in this case) varies when the points on the graphs are altered. The contour plot on the other hand shows whether the selected ranges of factors are nearing their individual or combined optimal points. An optimal point is indicated by the presence of a circle or oval shaped contour line, as can be seen in Figure 2. This indicates that the range of Fe^{3+} and Cu^{2+} must be altered in order to identify the optimal points. It is best when the interception of midpoint lines falls within this circle, since this implies that the ranges of factor are chosen correctly to represent the entire optimal and near optimal conditions. Modifications to the range of factors are best done only for those factors that have significant effect on the response in order to avoid complications in setting the ranges of factors.

DETERMINATION OF OPTIMAL LEVELS OF IONS CONCENTRATIONS FOR LIPID PRODUCTION

The highest amount of lipid produced in this study was 38.66% g/g biomass. This level was achieved when the concentration of Mg^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} were at 5.00, 0.017, 0.000 and 0.0005 g/L, respectively. The percentage of increment in lipid yield is 32.22%, where the initial yield before optimization process was 29.24% g/g biomass.

The polynomial equation stated above can be useful to estimate the amount of lipid that would be produced in cases of attempts to alter the concentrations of these four ions in order to increase productivity beyond the maximum yield in this study. Since it has been previously

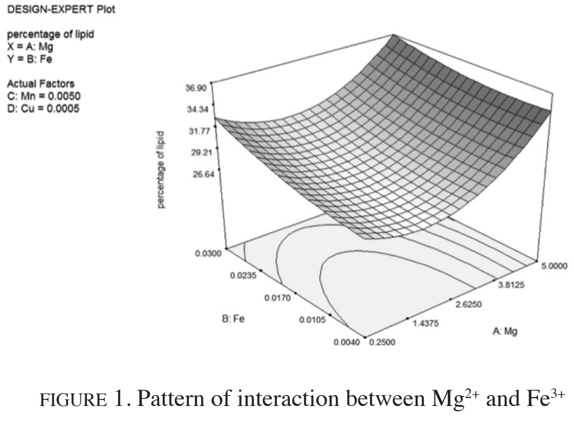

FIGURE 1. Pattern of interaction between Mg^{2+} and Fe^{3+}

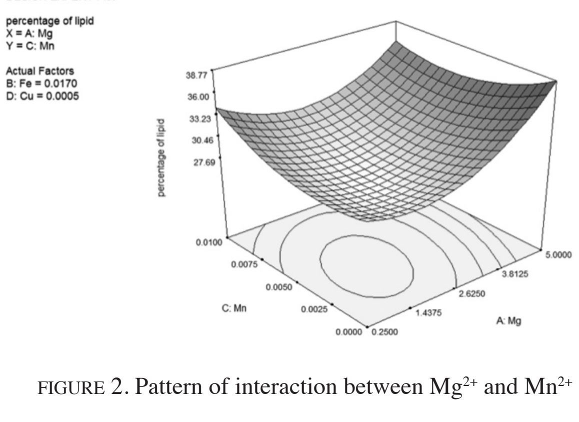

FIGURE 2. Pattern of interaction between Mg^{2+} and Mn^{2+}

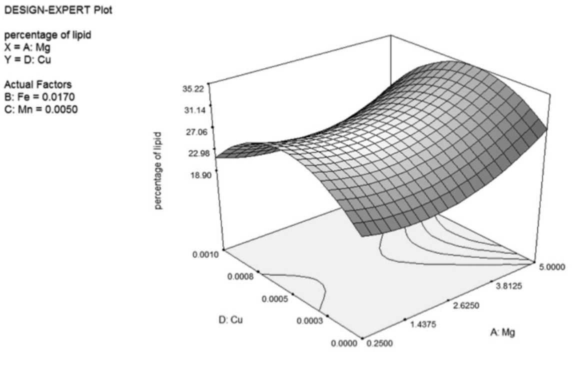

FIGURE 3. Pattern of interaction between Mg^{2+} and Cu^{2+}

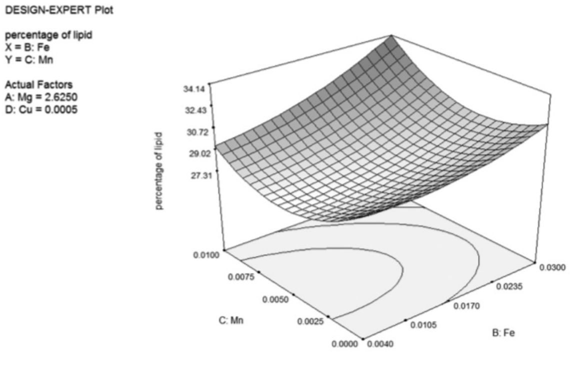

FIGURE 4. Pattern of interaction between Fe^{3+} and Mn^{2+}

proven that increased magnesium ion concentration has positive effect on lipid accumulation process in various fungi (Farhila et al. 2008; Lilly 1965), efforts to further promote lipid accumulation in this fungus can be emphasized on the concentration of Mg^{2+}. This is due to the possible role played by magnesium ion as an important cofactor for the enzymes involved in microbial lipogenesis, such as malic enzyme, fatty acid synthase and ATP citrate lyase (Farhila et al. 2008) and also in promoting the growth of mycelia (Lilly 1965).

Special care must be taken to ensure that the spore suspension is made from fresh and active spores. In our study, we noticed that those spores kept for more than one week in fridge (4ºC) take slightly longer time to germinate, compared to the fresh ones. This is particularly crucial for studies that are intended to maximize the production of lipid or similar metabolites in fungi.

CONCLUSION

The statistical analysis revealed that increasing Mg^{2+} and Fe^{3+}concentrations up to 5 g/L and 0.03 g/L respectively has significant positive effects on the lipid accumulation process of *C. bainieri* 2A1. Mn^{2+} and Cu^{2+} in combination have significant negative effects on the process. Lowering the concentration of these ions to their respective critical levels might lead to improved lipid accumulation.

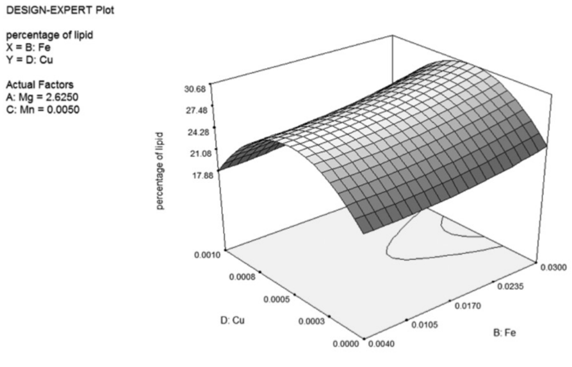

FIGURE 5. Pattern of interaction between Fe^{3+} and Cu^{2+}

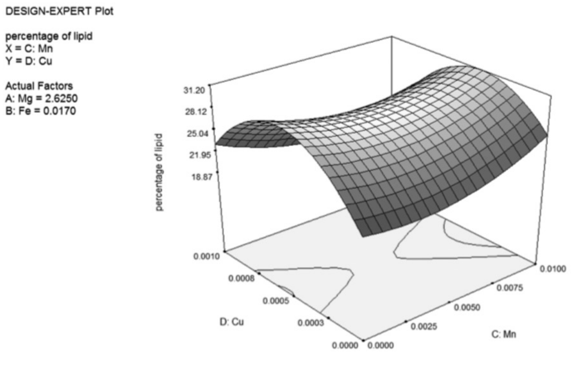

FIGURE 6. Pattern of interaction between Mn^{2+} and Cu^{2+}

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