Optimization of *Aspergillus niger* Nutritional Conditions using Statistical Experimental Methods for Bio-Recovery of Manganese from Pyrolusite

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Summary: The nutritional requirements for *Aspergillus niger* PCSIR-06 for bio-recovery of manganese from pyrolusite ore were optimized. Box-Bhenken design and response surface methodology were used for designing of experiment and statistical analysis of the results. This procedure limited the number of actual experiments to 54 for studying the possible interaction between six nutrients. The optimum concentration of the nutrients were Sucrose 148.5 g/L, KH_2PO_4 0.50 g/L, NH_4NO_3 0.33 g/L, $MgSO_4$ 0.41 g/L, Zn 23.76 mg/L, Fe 0.18 mg/L for *Aspergillus niger* to achieve maximum bio-recovery of manganese (82.47 ± 5.67%). The verification run confirmed the predicted optimized concentration of all the six ingredients for maximum bioleaching of manganese and successfully confirmed the use of Box-Bhenken experimental design for maximum bio-recovery. Results also revealed that small and less time consuming experimental designs could be efficient for optimization of bio-recovery processes.

Introduction

Manganese is an essential element in people's daily food consumption and has several industrial uses. Manganese is used in steel alloys to increase many favourable characteristics such as strength, hardness, and durability. In fact steel cannot be produced without manganese; it is an essential ingredient in the process. Thus, it is aptly called the Achilles heel of the iron and steel industry [1]. Manganese is also used to colour glass an amethyst colour. That is not so surprising since manganese is the trace element responsible for quartz's purple variety, amethyst.

Manganese constitutes 0.10% of earth crust by weight. The major accumulating minerals of manganese are oxides, carbonates, and silicates. The most important being psiomelane, pyrousite, manganite, rhodochrosite, chondrite, *etc*. The manganese ores are mostly classified into three grades, high (44-48% Mn), medium (35-44% Mn) and low grade (24-35% Mn) [2]. Recovery of metals from ores of low metal contents using pyro-metallurgical and hydrometallurgical processes is expensive due to high energy and capital costs. The major problem is the rising costs of environmental protection. The use of microorganism for the recovery of metals from low grade is considered a better alternative because of low costs and of reduced environmental pollution. There are three groups of microorganisms, autotrophic bacteria [3-5], heterotrophic bacteria [6] and fungi [7] involved in metal recovery processes. The microbial leaching of manganese is generally carried out by using heterotrophic microorganisms which live in micro-aerobic conditions and require organic nutrient to serve as energy and carbon source for their growth.

In development of industrial processes the optimization of conditions has vital importance. One variable at a time (OVAT), a classical optimization methodology is most frequently used. It is time and effort consuming and lacks the study of interaction between variables [8]. In order to optimise biotechnology processes, the statistical approaches provide cost-effective solution as they provide preplanned methods through which interaction between variables can easily be calculated [8-9]. Response surface methodology (RSM) is one of the useful models for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out limited number of experiments. The present study was designed to optimize the nutrient concentration that play important role in growth of fungus and bio-recovery of manganese. Thus to study the main and interaction effects of the different medium nutrient factors on bio-recovery of manganese, Box-Behnken design [10] and response surface methodology [11,12] were applied.

Results and Discussion

The application of response surface methodology to optimize the medium composition

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for various processes has been reported by many workers [13-17]. In order to determine the optimum response region for manganese bioleaching the independent variables Sucrose, KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn, and Fe were studied in three levels. The results of bio-recovery of manganese in 54-trial design matrix of Box-Behnken design [10] are shown in Table-1.

Table-1: Observed, predicted response and residual values in different runs.

S. No.	Run No.	Bio-recovery of Manganese, %		Residual value
		Actual Response	Predicted response	
1	24	42.60	42.60	0.00
2	18	42.60	42.60	0.00
3	47	42.60	42.60	0.00
4	13	42.60	42.60	0.00
5	50	42.60	42.60	0.00
6	36	42.60	42.60	0.00
7	48	16.57	22.04	-5.46
8	38	69.50	74.89	-5.39
9	33	21.29	18.39	2.91
10	22	60.25	60.68	-0.43
11	54	14.625	15.64	-1.02
12	27	71.92	73.38	-1.46
13	6	21.20	17.26	3.94
14	40	71.35	64.44	6.91
15	30	39.60	42.92	-3.32
16	17	38.88	43.87	-4.99
17	5	58.21	55.50	2.71
18	20	43.20	47.93	-4.73
19	51	59.30	51.67	7.63
20	42	41.04	46.65	-5.61
21	44	58.00	50.11	7.89
22	31	36.97	36.56	0.42
23	49	43.20	37.00	6.20
24	19	45.36	43.47	1.89
25	3	38.88	37.48	1.40
26	2	32.40	35.19	-2.79
27	35	38.88	38.32	0.56
28	52	43.92	43.09	0.83
29	21	39.83	43.95	-4.12
30	46	36.00	39.97	-3.97
31	11	24.50	24.36	0.14
32	7	69.58	67.70	1.88
33	16	20.99	20.01	0.98
34	26	70.40	68.24	2.16
35	43	14.62	18.24	-3.61
36	32	70.50	70.04	0.46
37	28	14.62	15.06	-0.44
38	53	70.16	71.75	-1.59
39	34	45.54	43.76	1.78
40	25	36.48	44.67	-8.19
41	39	38.88	50.43	-11.55
42	15	45.36	45.37	-0.01
43	14	58.92	56.02	2.90
44	10	57.15	48.49	8.66
45	8	58.00	52.71	5.29
46	45	40.32	39.20	1.12
47	12	14.62	10.77	3.85
48	37	70.50	69.82	0.68
49	23	21.71	18.66	3.06
50	1	69.80	66.12	3.68
51	9	16.46	17.91	-1.45
52	41	69.65	70.48	-0.83
53	29	21.20	24.11	-2.91
54	4	59.00	65.08	-6.08

Model Diagnostics

Residual analysis (Fig. 1) was used to examine the adequacy of the presented model. The normal probability plot in Fig. 1a which is used to check the violation of normality assumption show no serious violation as the data points following close to the straight line. Fig. 1b is used for testing the assumption of constant variance. The constant range of residuals across the graph show that the model need no transformation. Fig. 1c checks the lurking variables that may have influenced the response during the experiment. The plot should show a random scatter as in Fig. 1b. Trends indicate a time related variable lurking in the background. Blocking and randomization provide insurance against trends ruining the analysis. The residual differences were large enough when compared with the actual and predicted one. The difference was due to the presence of more noise during the experimentation. Fig. 1d represents the difference between the actual and predicted response. Box-Cox plot provides a guide line for selecting the correct power law transformation. The lowest point on Box-Cox plot represents the value of lambda (λ) that results in the minimum residual sum of squares in the transformed model. Here the value of Lambda is 'λ =1' which means no transformation is required (Fig. 1e).

Response Analysis

The bio-recovery of manganese as response in Table-1 was correlated using quadratic model

$$Y = \beta_o+\beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_4X_4+ \beta_5X_5 + \beta_6X_6 + \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{15}X_1X_5+ \beta_{16}X_1X_6+ \beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{25}X_2X_5+ \beta_{26}X_2X_6+ \beta_{34}X_3X_4+ \beta_{35}X_3X_5+ \beta_{36}X_3X_6+ \beta_{45}X_4X_5+ \beta_{46}X_4X_6+ \beta_{56}X_5X_6+ \beta_{11}X_1^2+ \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{55}X_5^2 + \beta_{66}X_6^2$$

where Y is the measured response "bio-recovery of manganese", β_o is the model constant, β_1, β_2,.... β_6 are linear coefficient, β_{11}, β_{22}, β_{33} β_{44}, β_{55}, β_{66} are quadratic coefficient, $\beta_{12,}$ $\beta_{13,....}$ β_{46} are cross product coefficient and X_1, X_2, X_3 are coded independent variables. The model obtained by statistical package "Design expert" software describing the response as function of variable is as follows:

$$\text{Bio-recovery of Manganese} = 42.60 +24.26X_1-3.98X_2+0.71X_3 - 1.49X_4 - 0.65X_5 + 1.44X_6 - 0.1392X_1X_2-3.15X_1X_3 + 2.47X_1X_4+ 2.12X_1X_5 - 1.37X_1X_6-2.13X_2X_3+3.18X_2X_4-1.49X_2X_5- 2.11X_2X_6 - 2.19X_3X_4 - 3.54X_3X_5 - 0.30X_3X_6+ 0.29X_4X_5+ 1.29X_4X_6 - 2.50X_5X_6 + 1.35X_1^2+ 3.36X_2^2 - 1.00X_3^2 - 1.47X_4^2 + 1.94X_5^2 - 0.32X_6^2$$

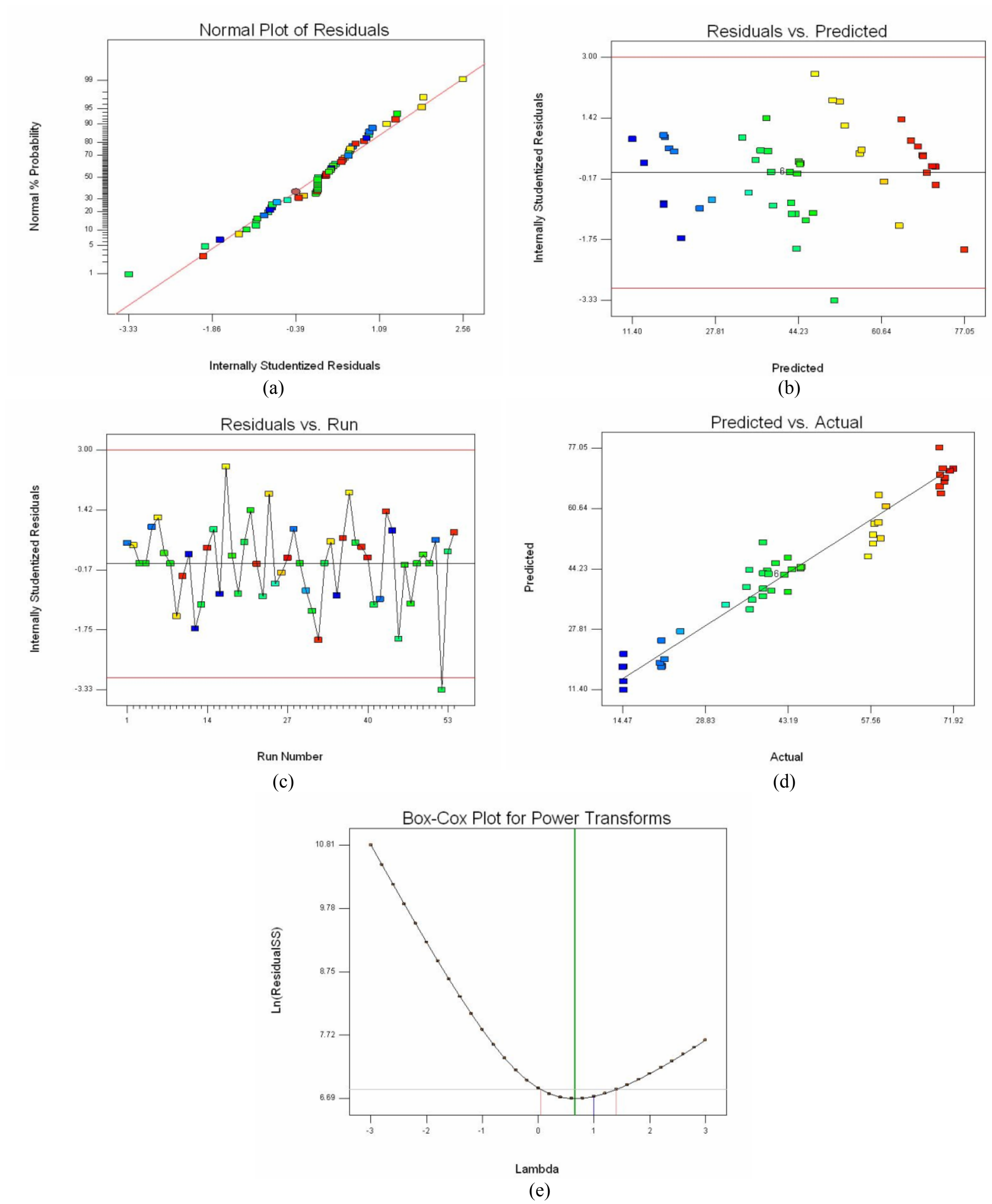


Fig. 1: Residual diagnostic of model for bioleaching of manganese
(a) Normal probability versus residuals (b) Residual versus predicted
(c) Residual versus run order (d) Predicted versus actual
(e) Box-Cox Plot for power transformation

The results of 2^{nd} order response surface model fitted in the form of analysis of variance (ANOVA) are depicted in Table-2. The Model F-value of 18.09 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob>F" less than 0.0500 indicate model terms are significant. In this case X_1 and X_2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. As there are many insignificant model terms, model reduction could improve the model. The "Pred R-Squared" of 0.7360 is in reasonable agreement with the "Adj R-Squared" of 0.8970. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 16.113 indicates an adequate signal. This indicates a better precision and reliability of the experiment.

Table-2: Analysis of variance (ANOVA) for the quadratic model for response bio-recovery of manganese.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-value Prob > F
Model	15644.15	27.00	579.41	18.0942	<0.0001
X_1	14121.69	1.00	14121.69	441.0000	<0.0001
X_2	380.54	1.00	380.54	11.8838	0.0019
X_3	11.95	1.00	11.95	0.3732	0.5466
X_4	53.39	1.00	53.39	1.6676	0.2079
X_5	10.22	1.00	10.22	0.3193	0.5769
X_6	49.86	1.00	49.86	1.5571	0.2232
X_1X_2	00.15	1.00	00.15	0.0048	0.9450
X_1X_3	79.23	1.00	79.23	2.4743	0.1278
X_1X_4	97.75	1.00	97.75	3.0528	0.0924
X_1X_5	35.80	1.00	35.80	1.1182	0.3000
X_1X_6	15.05	1.00	15.05	0.4701	0.4990
X_2X_3	36.34	1.00	36.34	1.1349	0.2965
X_2X_4	116.55	1.00	116.55	3.6399	0.0675
X_2X_5	35.76	1.00	35.76	1.1168	0.3003
X_2X_6	35.57	1.00	35.57	1.1109	0.3016
X_3X_4	38.32	1.00	38.32	1.1968	0.2840
X_3X_5	100.04	1.00	100.04	3.1243	0.0889
X_3X_6	1.42	1.00	1.42	0.0446	0.8343
X_4X_5	0.69	1.00	0.69	0.0216	0.8842
X_4X_6	13.28	1.00	13.28	0.4149	0.5251
X_5X_6	49.85	1.00	49.85	1.5567	0.2233
X_1^2	18.61	1.00	18.61	0.5812	0.4527
X_2^2	116.15	1.00	116.15	3.6274	0.0680
X_3^2	10.33	1.00	10.33	0.3228	0.5748
X_4^2	22.11	1.00	22.11	0.6907	0.4135
X_5^2	38.83	1.00	38.83	1.2128	0.2809
X_6^2	1.06	1.00	1.06	0.0334	0.8564
Residual	832.57	26.00	32.02		
Lack of Fit	832.57	21.00	39.64		
Pure Error	0.00	5.00	0.00		
Cor Total	16476.72	53.00			

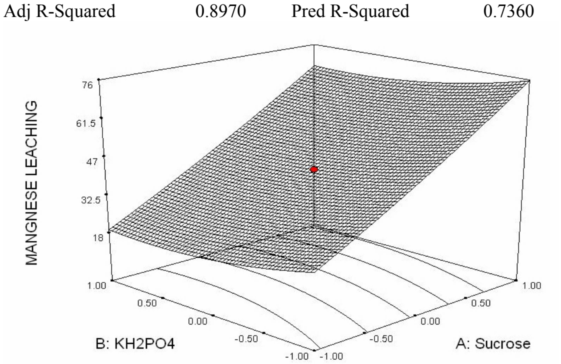
St. Dev. 5.6588 Mean 44.3144 C.V. % 12.76
PRESS 4349.76 Adeq Precision 16.1126 R-Squared 0.9495
Adj R-Squared 0.8970 Pred R-Squared 0.7360

As stated earlier that p-values of "prob > F" less than 0.0500 indicate significant model terms. Here in this particular case X_1 and X_2 are significant model terms. The "lack of fit value" of 39.64 is significant relative to pure error. A reduced model obtained describing the response as function of the most significant variables is as follows:

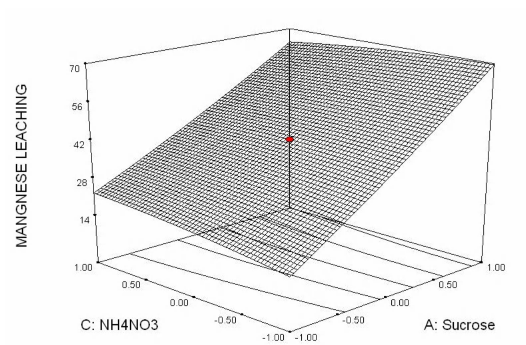
Bio-recovery of Manganese = $42.60 + 24.26X_1 - 3.98X_2$

The relationship of controlled experimental factors and measured responses on the basis of different selected criteria are shown in Figs. 2-5.

The effect of combination of sucrose with other factors *i.e.,* KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn and Fe, in the form of response surface and contour plots are presented in Fig. 2. The variation in concentration of KH_2PO_4 & sucrose, NH_4NO_3 & Sucrose keeping the other components at centre point, showed that maximum bio-recovery is possible if KH_2PO_4 & NH_4NO_3 are kept at minimum where as sucrose at maximum level. $MgSO_4$, Zn and iron had no pronounced significant effect on bio-recovery of manganese with their variations. Maximum bio-recovery was observed with maximum level of sucrose & KH_2PO_4 keeping the other factors at central point *i.e.,* 76%. The data revealed that all the medium components under investigation had direct relation with sucrose.



(a)



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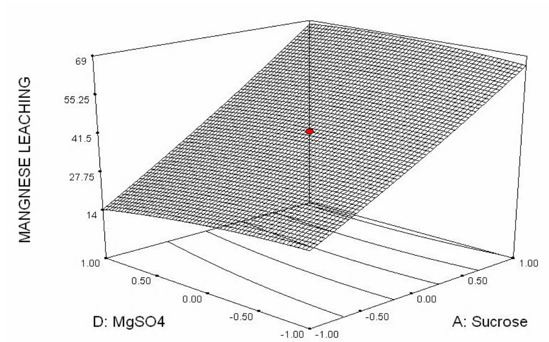

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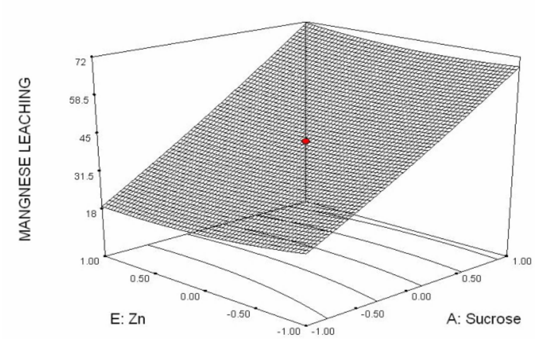
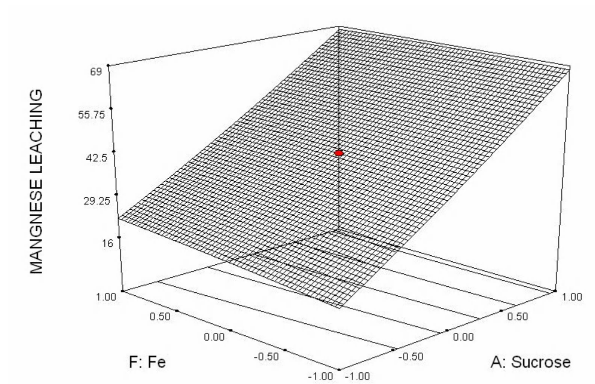


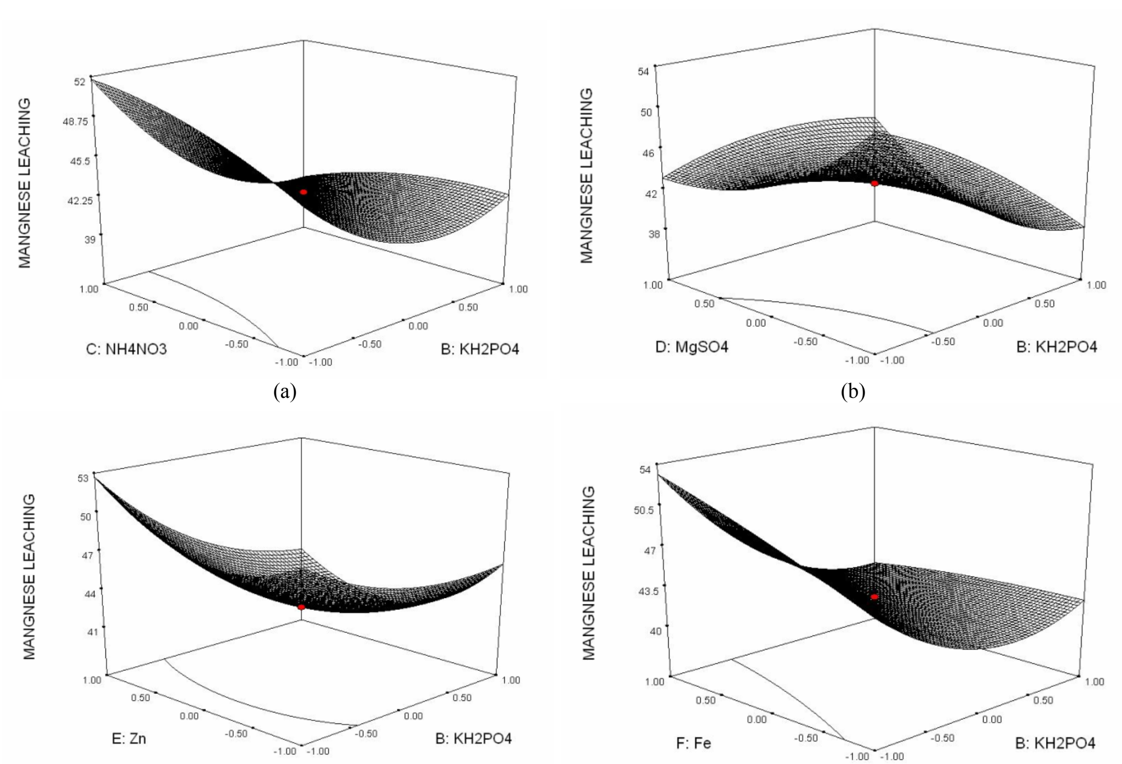
(d)



(e)

Fig. 2 Response surface and contour plot showing effect of sucrose in combination with other nutrient factors on bio-recovery of manganese from pyrolusite using *Aspergillus niger* PCSIR-06

a) Sucrose + KH_2PO_4 b) Sucrose + NH_4NO_3 c) Sucrose + $MgSO_4$,
d) Sucrose + Zn e) Sucrose + Fe



(a) (b) (c) (d)

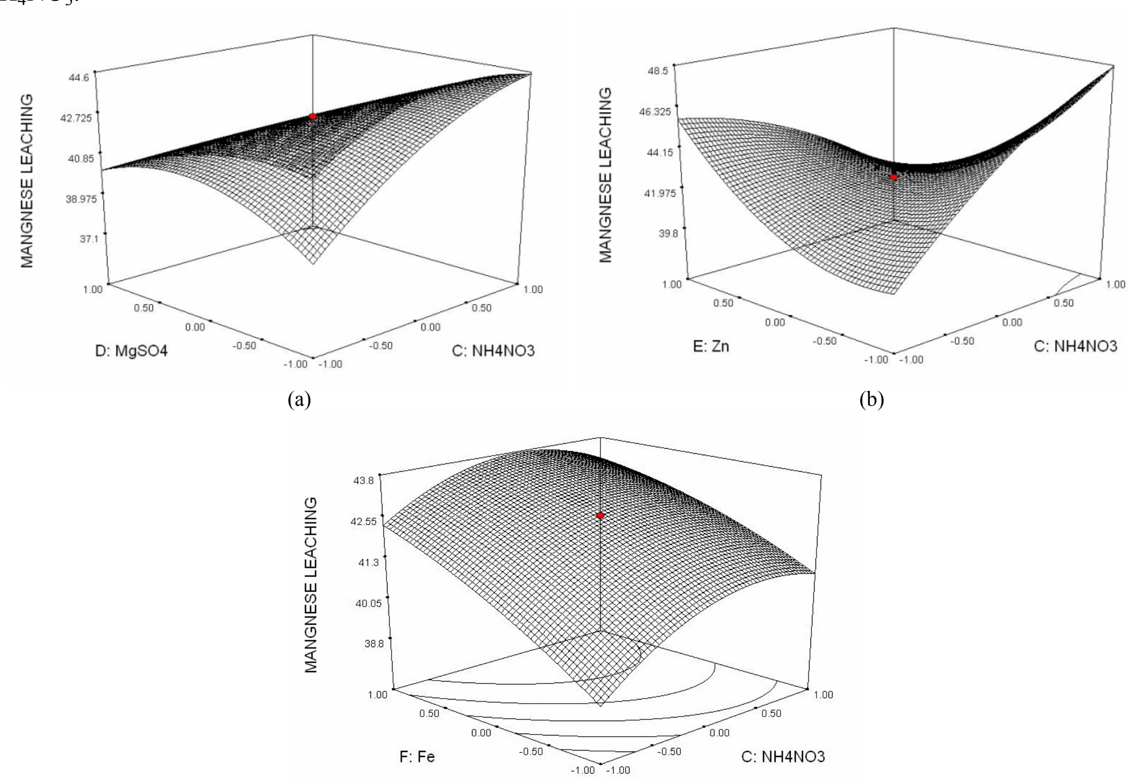
Fig. 3 Response surface and contour plot showing effect of sucrose in combination with other nutrient factors on bio-recovery of manganese from pyrolusite using *Aspergillus niger* PCSIR-06

a) KH_2PO_4 + NH_4NO_3 b) KH_2PO_4 + $MgSO_4$, c) KH_2PO_4 + Zn
d) KH_2PO_4 + Fe

The data regarding the combined variation in concentration in combinations of KH_2PO_4 with other nutrients keeping the remaining nutrients at central points is presented in Fig. 3. The data revealed that maximum bio-recovery of manganese achieved was about 53% in combinations of KH_2PO_4 at minimum with NH_4NO_3, Zn and iron at maximum level. The Fig. 3b show that for maximum bio-recovery of manganese $MgSO_4$ and KH_2PO_4 are required at minimum level while the other nutrients kept at centre point.

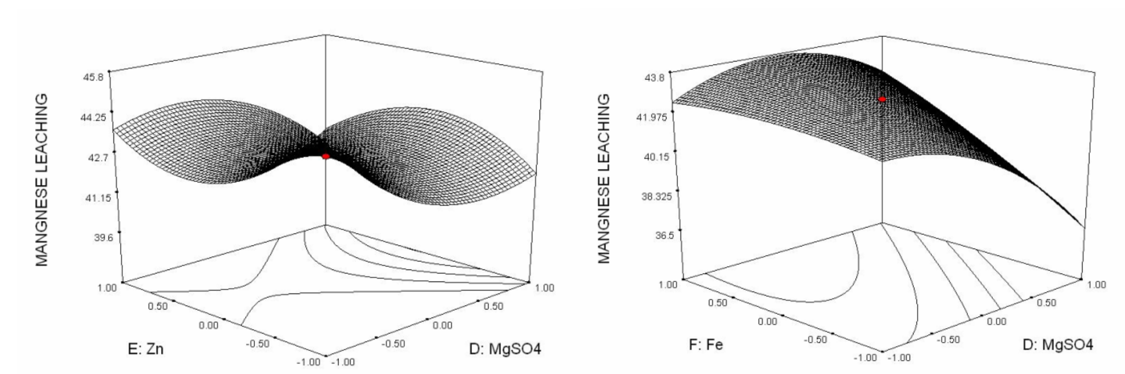
The response surface and contour plot of the effects of NH_4NO_3 concentration in combination with other nutrients *i.e.,* $MgSO_4$, Zn and Fe revealed that maximum bio-recovery of manganese was observed in the range of $\simeq$ 39.0% to $\simeq$ 45%. The variation in concentration of NH_4NO_3 along with $MgSO_4$ and Zn revealed that maximum level of NH_4NO_3 with minimum level of $MgSO_4$ and Zn are required for maximum bio-recovery of manganese while Fe is required at maximum level with central level of NH_4NO_3.

The data presented in Fig. 5 regarding variation in $MgSO_4$, Zn and Fe show that Zn at maximum level with $MgSO_4$ at minimum level are required for maximum manganese bio-recovery whereas, Fe with slightly higher level than centre point are needed by the *Aspergillus niger* PCSIR 06 for leaching of 43.8% manganese from pyrolusite ore.



(a) (b) (c)

Fig. 4 Response surface and contour plot showing effect of NH_4NO_3 in combination with other nutrient factors on bio-recovery of manganese from pyrolusite using *Aspergillus niger* PCSIR-06
a) $NH_4NO_3 + MgSO_4$, b) NH_4NO_3 + Zn c) NH_4NO_3 + Fe



(a) (b)

Fig. 5 Response surface and contour plot showing effect of $MgSO_4$ in combination with other nutrient factors on bio-recovery of manganese from pyrolusite using *Aspergillus niger* PCSIR-06
a) $MgSO_4$,+Zn b) $MgSO_4$ + Fe

The studies on optimization of Zn and iron keeping other nutrients at central point (Sucrose, NH_4NO_3, KH_2PO_4, $MgSO_4$) revealed that maximum bio-recovery of manganese could be achieved when Zn is kept at minimum and Fe at Maximum level (Fig. 6).

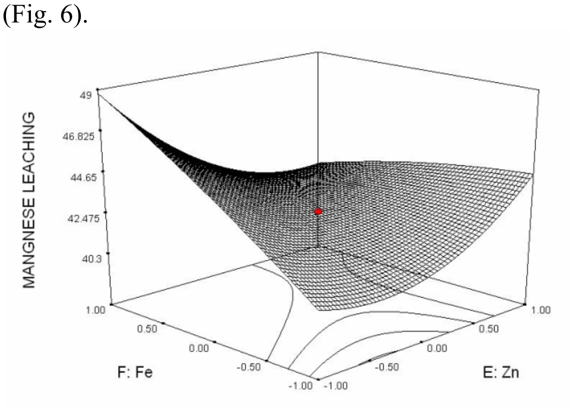


Fig.6 Response surface and contour plot showing effect of Fe and Zn on bioleaching of manganese from pyrolusite using *Aspergillus niger* PCSIR-06.

On the basis of above described relationships among the experimental factors, the Model predicted optimized concentrations of medium nutrients *i.e.,* Sucrose 148.5 g/L, KH_2PO_4 0.50 g/L, NH_4NO_3 0.33 g/L, $MgSO_4$ 0.41 g/L, Zn 23.76 mg/L, Fe 0.18 mg/L for maximum bio-recovery of manganese *i.e.,* 82.47 ± 5.67%.

Verification

A verification run was conducted in triplicate to confirm the predicted optimized concentrations of the medium nutrients using *Aspergillus niger* PCSIR 06 with requisite conditions. The mean bio-recovery of manganese of 85.69 ± 6.98 % was obtained with the optimized medium which is quite close to predicted production of 82.47 ± 5.67%.

Experimental

Ore

Manganese ore was collected from Wadh area of Khuzdar District, Balochistan, Pakistan. The ore assayed manganese content 24.57% (38.87% as MnO_2). The other contaminants (%) were Fe 3.49, Ca 0.75, Mg 0.03, Zn 0.01, Cu 0.027, Ni 0.03, S 0.16, P 0.23, Si 11.37 and LOI 10.16. The ore was ground to mesh size -150 and used throughout the studies.

Microorganism

Aspergillus niger PCSIR-06 isolated by Rahman *et al.* [18] was used in the studies.

Bioleaching Studies

Manganese bio-recovery experiments were carried out using sterilized mineral salt medium containing known quantities of ore in TUNAIR^{TM} Cell growth Shake Flask System, Shelton Scientific-IBI, working volume 1.0 liter shaking speed 200 rpm and filter size 0.22 micron nitrocellulose.

The ore pulp density was set at 1% w/v with maximum recovery of manganese [18].

At the end of each experiment, the leaching vessel was sterilized and final pH was measured. The analysis of the leached solution was carried out after filtration through Whatman No. 1 filter paper. The contents were filtered. The biomass and the ore remained on filter paper were washed with de-ionized water and with dilute sulphuric acid. The washings were added to the filtrate. The overall manganese extraction was determined at the end of each experiment.

Manganese contents in the recovered solution were determined by Atomic Absorption Spectrophotometer Model M series and using SOLAAR Software manufactured by Thermo Electron corporation UK using standard conditions, wavelength 279.5 nm, band pass 0.3 nm, lamp current 75 %, flame air/acetylene, fuel flow 1.0 L/min, burner type 50mm and burner height 7.0 mm.

Experimental Design and Optimization by RSM

Response surface methodology is a group of empirical techniques to evaluate relationship between cluster of controlled experimental factors and measured responses according to one or more selected criteria. The variables selected were Sucrose, KH_2PO_4, NH_4NO_3, $MgSO_4$, Zn, and Fe. To describe the nature of the response surface in the experimental region and to identify the optimum conditions for bio-recovery of manganese, a Box-Behnken design [10] was applied.

Table-3 presents the design matrix consisting of 52 trials to study the most significant variables affecting bio-recovery of manganese. Each variable was studied on three levels, coded -1, 0 and +1 for low, middle and high values. The actual and coded values along with units for different variables are shown in Table-4.

Table-3: Box-Behnken design of six factors coded and actual values.

S. No.	Run No.	Coded values						Actual values					
		X_1	X_2	X_3	X_4	X_5	X_6	X_1 (gl^{-1})	X_2 (gl^{-1})	X_3 (gl^{-1})	X_4 (gl^{-1})	X_5 (gl^{-1})	X_6 (gl^{-1})
1	24	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
2	18	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
3	47	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
4	13	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
5	50	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
6	36	0	0	0	0	0	0	100.00	2.50	2.50	0.25	12.00	0.40
7	48	-1	-1	0	-1	0	0	50.00	0.50	2.50	0.05	12.00	0.40
8	38	1	-1	0	-1	0	0	150.00	0.50	2.50	0.05	12.00	0.40
9	33	-1	1	0	-1	0	0	50.00	5.00	2.50	0.05	12.00	0.40
10	22	1	1	0	-1	0	0	150.00	5.00	2.50	0.05	12.00	0.40
11	54	-1	-1	0	1	0	0	50.00	0.50	2.50	0.50	12.00	0.40
12	27	1	-1	0	1	0	0	150.00	0.50	2.50	0.50	12.00	0.40
13	6	-1	1	0	1	0	0	50.00	5.00	2.50	0.50	12.00	0.40
14	40	1	1	0	1	0	0	150.00	5.00	2.50	0.50	12.00	0.40
15	30	0	-1	-1	0	-1	0	100.00	0.50	0.50	0.25	00.00	0.40
16	17	0	1	-1	0	-1	0	100.00	5.00	0.50	0.25	00.00	0.40
17	5	0	-1	1	0	-1	0	100.00	0.50	5.00	0.25	00.00	0.40
18	20	0	1	1	0	-1	0	100.00	5.00	5.00	0.25	00.00	0.40
19	51	0	-1	-1	0	1	0	100.00	0.50	0.50	0.25	24.00	0.40
20	42	0	1	-1	0	1	0	100.00	5.00	0.50	0.25	24.00	0.40
21	44	0	-1	1	0	1	0	100.00	0.50	5.00	0.25	24.00	0.40
22	31	0	1	1	0	1	0	100.00	5.00	5.00	0.25	24.00	0.40
23	49	0	0	-1	-1	0	-1	100.00	2.50	0.50	0.05	12.00	0.00
24	19	0	0	1	-1	0	-1	100.00	2.50	5.00	0.05	12.00	0.00
25	3	0	0	-1	1	0	-1	100.00	2.50	0.50	0.50	12.00	0.00
26	2	0	0	1	1	0	-1	100.00	2.50	5.00	0.50	12.00	0.00
27	35	0	0	-1	-1	0	1	100.00	2.50	0.50	0.05	12.00	8.00
28	52	0	0	1	-1	0	1	100.00	2.50	5.00	0.05	12.00	8.00
29	21	0	0	-1	1	0	1	100.00	2.50	0.50	0.50	12.00	8.00
30	46	0	0	1	1	0	1	100.00	2.50	5.00	0.50	12.00	8.00
31	11	-1	0	0	-1	-1	0	50.00	2.50	2.50	0.05	00.00	0.40
32	7	1	0	0	-1	-1	0	150.00	2.50	2.50	0.05	00.00	0.40
33	16	-1	0	0	1	-1	0	50.00	2.50	2.50	0.50	00.00	0.40
34	26	1	0	0	1	-1	0	150.00	2.50	2.50	0.50	00.00	0.40
35	43	-1	0	0	-1	1	0	50.00	2.50	2.50	0.05	24.00	0.40
36	32	1	0	0	-1	1	0	150.00	2.50	2.50	0.05	24.00	0.40
37	28	-1	0	0	1	1	0	50.00	2.50	2.50	0.50	24.00	0.40
38	53	1	0	0	1	1	0	150.00	2.50	2.50	0.50	24.00	0.40
39	34	0	-1	0	0	-1	-1	100.00	0.50	2.50	0.25	00.00	0.00
40	25	0	1	0	0	-1	-1	100.00	5.00	2.50	0.25	00.00	0.00
41	39	0	-1	0	0	1	-1	100.00	0.50	2.50	0.25	24.00	0.00
42	15	0	1	0	0	1	-1	100.00	5.00	2.50	0.25	24.00	0.00
43	14	0	-1	0	0	-1	1	100.00	0.50	2.50	0.25	00.00	8.00
44	10	0	1	0	0	-1	1	100.00	5.00	2.50	0.25	00.00	8.00
45	8	0	-1	0	0	1	1	100.00	0.50	2.50	0.25	24.00	8.00
46	45	0	1	0	0	1	1	100.00	5.00	2.50	0.25	24.00	8.00
47	12	-1	0	-1	0	0	-1	50.00	2.50	0.50	0.25	12.00	0.00
48	37	1	0	-1	0	0	-1	150.00	2.50	0.50	0.25	12.00	0.00
49	23	-1	0	1	0	0	-1	50.00	2.50	5.00	0.25	12.00	0.00
50	1	1	0	1	0	0	-1	150.00	2.50	5.00	0.25	12.00	0.00
51	9	-1	0	-1	0	0	1	50.00	2.50	0.50	0.25	12.00	8.00
52	41	1	0	-1	0	0	1	150.00	2.50	0.50	0.25	12.00	8.00
53	29	-1	0	1	0	0	1	50.00	2.50	5.00	0.25	12.00	8.00
54	4	1	0	1	0	0	1	150.00	2.50	5.00	0.25	12.00	8.00

Table 4: The coded and actual values of the factors in Box-Behnken design

Factors	Name of factors	Units	Low Coded	Low Actual	Centre Coded	Centre Actual	High Coded	High Actual
X_1	Sucrose	gl^{-1}	-1	50.00	0	100.00	1	150.00
X_2	KH_2PO_4	gl^{-1}	-1	0.50	0	2.50	1	5.00
X_3	NH_4NO_3	gl^{-1}	-1	0.50	0	2.50	1	5.00
X_4	$MgSO_4$	gl^{-1}	-1	0.05	0	0.025	1	0.50
X_5	Zn	mgl^{-1}	-1	0.00	0	12.00	1	24.00
X_6	Fe	mgl^{-1}	-1	0.00	0	0.40	1	0.80

To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variable and the response (Bio-recovery of manganese). For six factors, the equation was

$$Y = \beta_o+\beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_4X_4+ \beta_5X_5 + \beta_6X_6 + \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{15}X_1X_5+ \beta_{16}X_1X_6+ \beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{25}X_2X_5+ \beta_{26}X_2X_6+ \beta_{34}X_3X_4+ \beta_{35}X_3X_5+ \beta_{36}X_3X_6+ \beta_{45}X_4X_5+ \beta_{46}X_4X_6+ \beta_{56}X_5X_6+ \beta_{11}X_1^2+ \beta_{22}X_2^2 + \beta_{33}X_3^2\ \beta_{44}X_4^2 + \beta_{55}X_5^2 + \beta_{66}X_6^2$$

where Y is the measured response, β_o is the model constant, β_1, β_2,.... β_6 are linear coefficient, β_{11}, β_{22}, β_{33}, β_{44}, β_{55}, β_{66} are quadratic coefficient, $\beta_{12,}\ \beta_{13,....}\ \beta_{46}$ are cross product coefficient and X_1, X_2, X_3 are coded independent variables. Software Design-Expert (V.7.1.6 trial, Statease, Minneapolis, 2008) was used for regression analysis of experimental data and graphical analysis. The quality of fit of the polynomial model equation was expressed by a coefficient of determination R^2.The optimal concentrations of critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of Analysis of Variance (ANOVA). All the experiments were performed in triplicate and the mean values were given.

Conclusion

It can be safely concluded from the present study that:

1. Box-Bhenken design and response surface analysis could be utilised for optimization of nutritional requirement of microorganisms used in bio-recovery processes.
2. Three dimensional response curves with contour curve are useful tools for visualizing the main effects and interaction of the factors.
3. fix number of experiments planned through experimental design could be sufficient for optimization of bio-recovery processes.
4. the methodology used in the present studies could be effectively utilised for any process, where effect analysis and interaction of different factors involved in the experiment.

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