HIBISCUS PUBLISHER

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index

JOBIMB VOL 5 NO 2 2017

Statistical Optimization of Hexavalent Molybdenum Reduction by *Serratia* sp. strain MIE2 using Central Composite Design (CCD)

Nor Farahim Aziz[1], Mohd Izuan Effendi Halmi[2*], Wan Lutfi Wan Johari[1]

[1]Department of Environmental Science, Faculty of Environment, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Department of Land Management, Faculty of Agriculture, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author: Mohd Izuan Effendi Bin Halmi
Department of Land Management,
Faculty of Agriculture,
University Putra Malaysia,
43400 UPM Serdang,
Selangor, Malaysia.
Email: m_izuaneffendi@upm.edu.my

HISTORY

Received: 27th July 2017
Received in revised form: 2nd August 2017
Accepted: 5th of November 2017

KEYWORDS

bioremediation
molybdenum
agriculture soil
isolation
RSM

ABSTRACT

The conversion of hexavalent molybdenum (Mo (VI)) to Mo-blue is a bioremediation technique which reduces the toxicity of molybdenum to a less toxic form by bacteria. The aim of this study is to determine the optimum conditions of significant parameters or variables that affect the reduction of Mo (VI) to Mo-blue by the local isolate identified as *Serratia* sp. strain MIE2. Response Surface Methodology (RSM) was used in this study to optimize the reduction process using Central Composite Design (CCD) as an optimization matrix. The optimum conditions predicted by RSM using the desirability function for the reduction process were 20 mM molybdate concentration, 3.95 mM phosphate, 6.25 pH and 25 g/L glucose and Mo-blue production occurred at the absorbance value of 20.5 at 865 nm. The validation of the predicted optimum points showed the Mo-blue production occurred at the absorbance value of 21.85 with a deviation around 6.6 % from the RSM predicted value.

INTRODUCTION

Molybdenum (Mo) comes from Greek word 'molybdos' that brings the meaning of lead-like [1]. Molybdenite (MoS_2), are present abundant in the environment and it is grouped with other compounds such as lead, graphite, galena and others [2]. Usually, human consumed Mo through food and water. Mo is an essential element for human and animal, but at higher dosages, it gives a bad effect on health [3]. Food and water that contain 100 mg/kg of Mo will show signs of toxicity. Contaminated source of food and water with Mo can cause diarrhea, anemia, and also a high level of uric acid in the blood. Gout is the example of illnesses caused by the high concentrations of uric acid in the blood [4-5]. Regulatory bodies had set up the maximum levels of Mo in drinking water at 0.07 mg/L and for areas near to mining sites, the maximum concentration of Mo is 0.1 mg/L. Mo effects on human health through long exposure at low concentrations of Mo compounds. However, ruminants such as cows and sheep are more exposed to molybdenum poisoning or molybdenum pollutant than human beings. Signs of molybdenum toxicity in animals include anemia, anorexia, profound diarrhea, joint abnormalities, osteoporosis, hair discoloration, reduced sexual activity and death [6].

Bioremediation technology is a biological method that has the possibility to break up or overcome the toxicity of various contaminants in the environment using microorganisms [7]. This technology is not expensive, safe and user-friendly compared to chemical and physical techniques [8]. Microbes have the capability to resist or eliminate heavy metal from the environment via the mechanism of extra and intracellular sequestration, biopreciptation, changing redox state through an enzymatic reaction, biosorption, transport mechanism and or chelation [9]. Since the last hundred years ago, reduction of Mo to Mo-blue by bacteria has been reported worldwide. *E. coli* K12 is the first bacterium that reduces Mo to Mo-blue [10]. This is followed by *T. ferrooxidans* in 1988 [11], *Enterobacter cloacae* strain 48 (EC 48) in 2000 [12]. Since then, many Mo-reducing bacteria have been reported [13-15]. The mechanism involves enzymatic reduction that changes the Mo (VI) in the presence of phosphate ions to Mo-blue [16].

Response Surface Methodology or known as RSM is an optimization tool that designs an experiment based on principles of statistics, identify the effects of several parameters and determine optimum conditions for the required responses [17]. This method is more effective compared to the traditional method; 'one-factor-at-a time' approach (OFAT) [18]. The traditional method is not suitable for optimizing parameters because it fails to determine the parameters that give rise to the optimum response from several parameters. It is because the eventual interaction among the parameter is not taken into account in such procedures, and thus consuming more time [19]. In examining multiple parameters, statistical methods are the best and versatile technique because it makes the process easily optimized with fewer experimental. RSM has few experimental designs, and the user must understand and choose the suitable design that can match with the studies [20].

In a previous work, we have successfully optimized and maximized the production molybdenum reduction using OFAT approach and Box–Behnken designs as an optimization matrix for response surface methodology [18]. In this work, we try to investigate the optimization of Mo (VI) reduction by strain MIE2 using the central composite design (CCD) as the optimization matrix. This study would provide the information about the best optimization matrix for optimization of molybdenum reduction using response surface methodology.

MATERIALS AND METHODS

Cultivation of the bacterium

Strain MIE2 was grown in 100 ml of Low Phosphate Media which contained sodium molybdate ($NaMoO_4.2H_2O$) (2.42 g/L), as a source of Mo (VI) and glucose (10g/L) as a carbon source. Other chemicals were $(NH_4)._2SO_4$ (3g/L), $MgSO_4.7H_2O$ (0.5 g/L), yeast extract (0.5 g/L), and Na_2HPO_4 (0.4 g/L) using 250 ml conical flasks [16]. The carbon source needs to be autoclaved separately. The pH of the media was adjusted to 7 to put the media under neutral conditions. Agitation of the cultures of the bacteria in LPM was carried out at room temperature and on an orbital shaker at 150 rpm, for 24 hours. The production of Mo-blue from the LPM was measured using a UV-Visible Spectrophotometer at 865 nm [21].

Optimization using central composite design (CCD)

A standard RSM called CCD was applied to obtain a quadratic model. Central Composite Design (CCD) is a second order design [22]. This pattern helps to optimize the effective variable and analyze the interaction between the variables with a minimal number of experiments. 30 different experiments with four significant variables resulted from Plackett Burman [18] were conducted according to the Central Composite Design (CCD) as depicted in **Table 1**. In this design, four independent variables were studied at two different levels and fit into 30 individual experiments. The reduction of Mo (VI) to Mo-blue was taken as the response. The software Design Expert Version 6.0.8.0 (State Ease Inc.) was used for regression and graphical analysis of the response surface contour plots and 3D of the data obtained. The values of "Prob> F" less than 0.0500 indicates model terms are significant and if the values are greater than 0.1000 indicate that the model terms are not significant.

The linear quadratic model with four variables were expressed as:

$$Y = \beta o + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXi^2 + \sum_{j=2}^{k-1} \beta ijXiXj$$

Where Y is the response to Mo reduction to Mo-blue, βo the intercept term, βi represented as the linear effect, βii the squared effect, and βij is the interaction effect.

RESULTS AND DISCUSSION

Central Composite Designs (CCD) for optimization of Mo-blue production

In CCD, the variation of Mo (VI) reduction to Mo-blue in 30 individual experiment ranged from 0.15 to 20.0 OD at 865nm (**Table** 1). The empirical relationship between the response and input variable (significant variable) was expressed by the following quadratic model;

Y (Mo blue production) = +19.26+ 0.14*A+ 0.27*B+ 2.03*C+ 0.70*D-2.70*A2-1.06*B2-3.73*C2 2.70*D2=0.94*A*B+2.37*A*C-0.67*A*2.06*B*0.58*B*D

Where Y represents the response (Mo-blue); A, B, C, and D are the coded variables which represent molybdate concentration, phosphate concentration, pH and sucrose concentration

Table 1. CCD matrix for experimental design and response using RSM.

Run	Factor 1: Molybdate concentration ($mmol^{-1}$)	Factor 2 :Phosphate ($mmol^{-1}$)	Factor 3: Ph	Factor 4: Sucrose (g/l)	Mo-blue
1	30	5	7.5	40	12.88
2	30	2.9	5	10	1.44
3	20	3.95	6.25	25	17.37
4	20	1.85	6.25	25	14.68
5	20	3.95	3.75	25	0.359
6	10	2.9	7.5	40	14.65
7	20	3.95	6.25	25	20
8	30	2.9	7.5	10	11.34
9	30	5	5	10	12.43
10	10	2.9	7.5	10	13.32
11	10	2.9	5	40	6.51
12	40	3.95	6.25	25	12.47
13	10	5	5	40	12.84
14	20	3.95	6.25	55	13.56
15	20	3.95	6.25	-5	0.3975
16	30	5	7.5	10	14.43
17	20	3.95	6.25	25	19.56
18	10	2.9	5	10	10.45
19	30	2.9	7.5	40	15.11
20	10	5	5	10	13.93
21	20	3.95	6.25	25	19.336
22	10	5	7.5	10	5.98
23	0	3.95	6.25	25	1.42
24	20	6.05	6.25	25	12.38
25	20	3.95	8.75	25	5.29
26	30	5	5	40	1.35
27	20	3.95	6.25	25	19.655
28	30	2.9	5	40	0.15
29	20	3.95	6.25	25	19.62
30	10	5	7.5	40	10.23

Table 2. Statistical analysis of CCD.

Source	Sum Of Squares	Df	Mean Square	F Value	Prob > F
Model	952.69	14	68.05	4.11	0.0051
A	0.46	1	0.46	0.028	0.8699
B	1.76	1	1.76	0.11	0.7488
C	98.83	1	98.83	5.97	0.0274
D	11.66	1	11.66	0.70	0.4145
A^2	*200.51*	1	200.51	12.12	0.0034
B^2	30.67	1	30.67	1.85	0.1935
C^2	382.40	1	382.40	23.11	0.0002
D^2	199.26	1	199.26	12.04	0.0034
Ab	14.06	1	14.06	0.85	0.3712
Ac	89.97	1	89.97	5.44	0.0341
Ad	7.16	1	7.16	0.43	0.5208
Bc	67.65	1	67.65	4.09	0.0614
Bd	5.45	1	5.45	0.33	0.5745
Cd	39.69	1	39.69	2.40	0.1423
Residual	248.25	15	16.55		
Lack Of Fit	243.74	10	24.37	27.08	0.0010
Pure Error	4.50	5	0.90		
Cor Total	1200.93	29			

Note: DF:degrees of freedom of variance.

From **Table 2**, the value of Prob >F which was lower than 0.05 in 95% confidence intervals values denotes that the model is significant in Mo-blue production. The p-value for the lack of fit is insignificant, so the model is acceptable. Besides that, the mean square error of pure error is less than the value of lack of fit which is 4.50. Analysis of variance (ANOVA) in **Table 3** shows the results of quadratic models in Mo-blue production. The value of R-squared is 0.7933, which is not close to 1.

Meanwhile, the value for the Adjusted R-Squared is 0.6004 and does not have good relations between the true value and expected or predicted values. The function of adjusted R-Squared value modifies the R-Squared value for the sample size and the number of terms in the mod. The adjusted R-Squared may be noticeably smaller than the R-Squared. The adjusted R-Squared value in CCD is lesser than the R-Squared. For Adeq Precision, the function measures the signal to noise ratio. The ratio which higher than four is classified as desirable. The value of Adeq precision in CCD is 6.603 thus indicating an adequate signal.

Table 3. Analysis of variance (ANOVA) for CCD result.

Sources	Value
Standard Deviation	4.07
Mean	11.10
C.V.	36.63
R-Squared	0.7933
Adj R-Squared	0.6004
Pred R-Squared	0. 1745
Adeq Precision	6.603

Determination of optimum point using desirability function

The maximal hexavalent molybdenum reduction was determined using the desirability function method. The optimal conditions predicted by the desirability function were as follows: 20 mM molybdate, 3.95 mM phosphate, pH of 6.25 and 25 g/L glucose with overall production occurred at the absorbance value of 20.5. To validate this optimal condition, an experiment was performed according to the predicted values obtained. The validation result showed that Mo-blue was formed at an absorbance value of 21.85 when measured at 865 nm compared with the predicted value of only 20.5 with a deviation of 6.6 %.

Interaction between various factors on molybdenum (Mo) reduction

The interaction between parameters to produce Mo-blue is shown in the form of 3D plots and surface contours. **Fig 1(a)** shows that the increasing of concentration of Molybdate and phosphate will reduce the reduction of Mo (VI) to Mo-blue. Mo-blue production is inhibited by the higher concentration of phosphate. The optimum concentration for both parameters is in the middle of the contour with Mo is 20 mM, and phosphate concentration is 3.95 mM. From **Fig. 1(b)** the bacteria cannot deal with too acidic and too alkali environments. The optimum pH that supported the production of Mo-blue is 6.25. **Fig. 1(c)** shows that carbon source plays an important role in the reduction of molybdenum. Higher concentration of glucose and Molybdate will inhibit the production of Mo-blue.

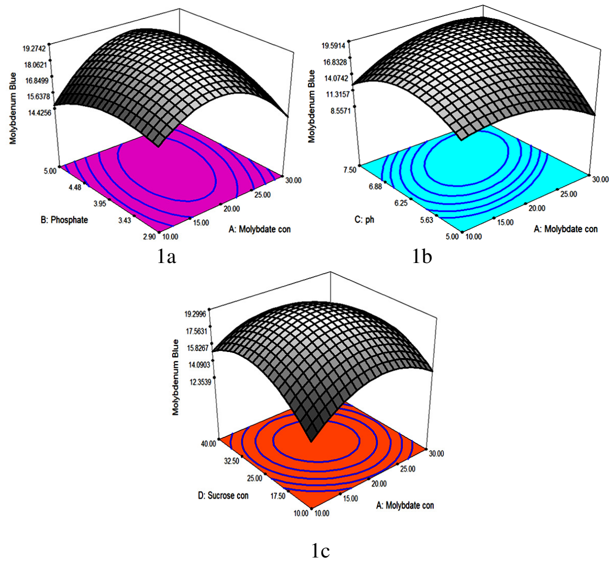

Fig. 1. Response surface curve plot.

CONCLUSION

From the analysis, response surface methodology (RSM) with Central Composite Design (CCD) as the optimization matrix was successfully applied in the optimization of Mo (VI) reduction by *Serratia* sp. MIE2. The optimal conditions predicted by RSM were 20 mM molybdate, 3.95 mM phosphate, pH 6.25 and 25 g/L glucose with the absorbance of 19.53 for Mo-blue production measured at 865 nm. The experimental validation of the predicted optimal conditions showed that the maximum Mo-blue production occurred at absorbance of 21.85, with a 6.6 % deviation from the predicted value obtained from RSM.

ACKNOWLEDGEMENTS

This project was supported by funds from the Ministry of Science, Technology, and Innovation (MOSTI) under the Science Fund Grant nos. 02-01-04-SF0445 and 02-01-04-SF0445.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Loyson P. Influences of ancient Greek on chemical terminology. J Chem Educ. 2009;86(10):1195.
2. Lennartson A. Made by molybdenum. Nature chemistry. 2014;6(8):746-.
3. Williams R, Da Silva JF. The involvement of molybdenum in life. Biochemical and biophysical research communications. 2002;292(2):293-9.
4. Vyskocil A, Viau C. Assessment of molybdenum toxicity in humans. Journal of Applied Toxicology. 1999;19(3):185-92.
5. WHO. Guidelines for drinking water quality. . World Health Organisation, Geneva. 1993(Second edition.).
6. Neunhäuserer C, Berreck M, Insam H. Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. Water, air, and soil pollution. 2001;128(1-2):85-96.
7. Halmi MIE, Abdullah SRS, Johari WLW, Ali MSM, Shaharuddin NA, Khalid A, et al. Modelling the kinetics of hexavalent molybdenum (Mo6+) reduction by the *Serratia* sp. strain MIE2 in batch culture. Rendiconti Lincei. 2016;27(4):653-63.

8. Fingerman M. Bioremediation of aquatic and terrestrial ecosystems: CRC Press; 2016.
9. Lemire J, Turner RJ. 10 Biochemical Pathways in Bacteria. Handbook of Metal-Microbe Interactions and Bioremediation. 2017:143.
10. Campbell AM, Del Campillo-Campbell A, Villaret DB. Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. Proceedings of the National Academy of Sciences. 1985;82(1):227-31.
11. Sugio T, Tsujita Y, Katagiri T, Inagaki K, Tano T. Reduction of Mo6+ with elemental sulfur by *Thiobacillus ferrooxidans*. Journal of Bacteriology. 1988;170(12):5956-9.
12. Shukor M, Shamaan N, Syed M, Lee C, Karim M. Characterization and quantification of molybdenum blue production in *Enterobacter cloacae* strain 48 using 12-molybdophosphate as the reference compound. Asia-Pacific Journal of Molecular Biology and Biotechnology. 2000;8(2):167-72.
13. Masdor N, Shukor MSA, Khan A, Halmi MIE, Abdullah SRS, Shamaan NA, et al. Isolation and characterization of a molybdenum-reducing and SDS-degrading *Klebsiella oxytoca* strain Aft-7 and its bioremediation application in the environment. Biodiversitas Journal of Biological Diversity. 2015;16(2).
14. Mansur R, Gusmanizar N, Dahalan FA, Masdor NA, Ahmad SA, Shukor MS, et al. Isolation and characterization of a molybdenum-reducing and amide-degrading *Burkholderia* sp. strain neni-11 in soils from west sumatera, Indonesia. The IIOAB Journal. 2016;7(1):28.
15. Ibrahim Y, Abdel-Mongy M, Shukor MS, Hussein S, Ling APK, Shukor MY. Characterization of a molybdenum-reducing bacterium with the ability to degrade phenol, isolated in soils from Egypt. BioTechnologia. Journal of Biotechnology Computational Biology and Bionanotechnology. 2015;96(3).
16. Ghani B, Takai M, Hisham NZ, Kishimoto N, Ismail AM, Tano T, et al. Isolation and characterization of a Mo^{6+}-reducing bacterium. Applied and Environmental Microbiology. 1993;59(4):1176-80.
17. Sanusi SNA, Halmi MIE, Abdullah SRS, Hassan HA, Hamzah FM, Idris M. Comparative process optimization of pilot-scale total petroleum hydrocarbon (TPH) degradation by *Paspalum scrobiculatum* L. Hack using response surface methodology (RSM) and artificial neural networks (ANNs). Ecological Engineering. 2016;97:524-34.
18. Halmi MIE, Abdullah SRS, Wasoh H, Johari WLW, bin Mohd Ali MS, Shaharuddin NA, et al. Optimization and maximization of hexavalent molybdenum reduction to Mo-blue by *Serratia* sp. strain MIE2 using response surface methodology. Rendiconti Lincei. 2016;27(4):697-709.
19. Mohammed N, Ahmad WA. Application of Response Surface Methodology (RSM) for optimizing removal of Cr (VI) wastewater using Cr (VI)-reducing biofilm systems. Malaysian Journal of Fundamental and Applied Sciences. 2014;6(1).
20. Ibrahim S, Shukor MY, Khalil KA, Halmi MIE, Syed MA, Ahmad SA. Application of response surface methodology for optimising caffeine-degrading parameters by *Leifsonia* sp. strain SIU. Journal of environmental biology. 2015;36(5):1215.
21. Shukor Y. Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. Journal of Environmental Microbiology and Toxicology. 2013;1(1).
22. Ghafari S, Aziz HA, Isa MH, Zinatizadeh AA. Application of response surface methodology (RSM) to optimize coagulation–flocculation treatment of leachate using poly-aluminum chloride (PAC) and alum. Journal of Hazardous Materials. 2009;163(2):650-6.