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Bioreduction of Hexavalent Molybdenum (Mo^{6+}) to Molybdenum-Blue by *Serratia* sp. strain MIE2 and Prediction of Optimum Points using Artificial Neural Network

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ABSTRACT

Molybdenum, a heavy metal, is toxic to ruminants and can inhibit animal spermatogenesis. A previously identified Mo-reducing bacterium known as *Serratia* sp. strain MIE2 was optimized using an Artificial neural network (ANN) to predict points that can give optimum molybdenum blue production to combat molybdenum pollution in agricultural soils. ANN predicted the best optimum points occurring at pH, temperature, sucrose, ammonium sulfate, phosphate, and molybdate concentrations of 6.5 to 7.0, between 27 to 35°C, 30 to 40 g/L, 10 g/L, between 4 and 6 mM and between 10 and 20 mM, respectively, with a Mo-blue production of 14 absorbance unit as measured at 865 nm. The effect of various xenobiotics such as carbofuran, diazinon, methomyl, malathion, trichlorfon, bendiocarb, carbaryl, hexane, and butanol showed minimal inhibition to molybdenum blue production. The results indicate ANN's utility in predicting optimum production of Mo blue from this bacterium.

INTRODUCTION

Because it is an important component in so many enzymes, molybdenum is an essential microelement for all living things. The most prevalent soluble form found in nature, molybdate (VI), is one of five potential oxidation states. The molybdate anion is the primary soluble form of molybdenum in solutions with a pH close to neutral; it is said to be the sole form that plants can absorb. Molybdate ions can be readily polymerized into $Mo_7O_{24}^{6-}$, $Mo_8O_{26}^{4-}$ and $Mo_{12}O_{37}^{2-}$-type polyions in acidic environments. Reducing agents can be used to decrease these polyions, resulting in the creation of "isopolymolybdenum blue." In addition, these polyions can form heteropolymolybdate by combining with specific heteroatoms, such as phosphate, arsenate, tungstate, sulphate, or silicate. These compounds can then be reduced chemically with reducing agents like dithionite or ascorbic acid or biologically with Mo-reducing enzymes to produce intense blue colloidal products called heteropolymolybdenum blue [1,2]. Molybdenum-containing wastes could be released to the ecosystem from their utilization as alloys, catalysts, and lubricants by mining activities, the application of sewage sludge, fertilizers, and atmospheric deposition. Typically, soil has 0.2–6 mg/kg of Mo, however soil that is polluted with metals might have values of 10-100 mg/kg. Cattle that graze on soil that is rich in molybdenum may be at risk of molybdenosis, a disease caused by a lack of copper, since molybdenum inhibits ruminants' ability to absorb copper [3]. The harmful effects of molybdenum on embryogenesis and spermatogenesis in mice and catfish have been demonstrated in recent studies, and levels as low as several parts per million are inhibited [4,5].

One method for cleaning up polluted environments is bioremediation. Agricultural soil polluted with molybdate in Tyrol, Austria, was the first to undergo molybdenum bioremediation with success [6]. The molybdate was able to be reduced in toxicity by the use of phytoremediation and a combination of soil bacteria that rendered it insoluble. Evidence of microbial reduction of molybdate has been documented in multiple studies [8-10].

Bacterial reduction processes, which are part of their detoxification strategy, include reducing molybdenum from its 6+ oxidation state to a lower oxidation state, such as Mo-blue. In their study, Shukor et al. shown that molybdenum-reducing bacteria undergo reduction by an enzymatic mechanism rather than a chemical mediator [7]. So far, no molybdenum-reducing bacteria have been found in agricultural soil, according to the literature. Since molybdate is hazardous to animals such as cattle and fish, a novel molybdenum reducer derived from agricultural soil is required for bioremediation. Because some crops are tolerant of and absorb a high molybdate concentration, untreated agricultural soil may not be suited for growing these crops. Ingesting plants that have built up molybdate in their tissues could be harmful to humans and other creatures [8,9].

The present work reports on the optimization using both OVAT or OFAT (one variable-at-a-time or one factor-at-a-time) and Artificial Neural Network (ANN) of a previously-isolated Mo-reducing bacterium [10]. The optimization process was performed using one variable at a time (OVAT) with molybdate concentration, phosphate concentration, pH, temperature, ammonium sulphate concentration, and sucrose concentration as variables and molybdenum blue as a response. We try to use an artificial neural network (ANN) to fit OVAT experimental data to predict the optimum condition. We tried to fit the experimental data with ANN to select the best parameter values that minimize the entire error over the set of data points being considered, thus predicting the best optimal points. The characterization of this bacterium would make it suitable for future bioremediation works on agricultural soil contaminated with molybdenum.

MATERIALS AND METHOD

Chemicals

All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Fisher (Fisher Scientific (M) Sdn Bhd, Shah Alam, Selangor, Malaysia) and Merck (Darmstadt, Germany).

Statistical analysis

The effect of toxicants was statistically assessed using GraphPad Prism, with three replicates. Artificial Neural Network (ANN) was analyzed using Automated Neural Network, STATISTICA 8.0, (Statsoft)

Growth of molybdenum-reducing bacterium

The bacterium was grown in low phosphate media containing 10 mM sodium molybdate for 24 hours. The ingredients of low phosphate media were glucose (1%), $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%), NaCl (0.5%), $Na_2MoO_4.2H_2O$ (0.24 %), Na_2HPO_4 (0.04%). 10 µl aliquot of the soil suspension was pipetted and spread onto agar of low phosphate media (pH 7.5). When blue colonies form, it means that the molybdenum-reducing bacterium is reducing molybdate [11]. In order to cultivate a pure culture, the colony with the highest blue intensity was chosen and moved to low phosphate media (LPM). In order to conduct the molybdate reduction experiment, 10 microliters of the bacterial culture were added to 10 milliliters of freshly made LPM. The next step was to incubate the mixture at 30°C for 24 hours so that the molybdate might be reduced. Later, 1 milliliter of the Mo-blue solution extracted from the LPM was taken and spun in an Eppendorf™ centrifuge at 10,000 x g for 20 minutes at room temperature. Following this, the liquid was examined using a UV-spectrophotometer (Shimadzu 1201), which scanned the 400 to 900 nm range, with LPM as the adjustment reference point [10]. Direct dilution with blank media was performed for absorbance values greater than 1.0.

Predictive optimization using Artificial Neural Network (ANN)

Low phosphate medium (LPM) as reported by Ghani et al. [11] was used to study the effect of various parameters such as molybdate and phosphate concentration, pH, electron donor sources, temperature, and nitrogen sources on molybdate reduction by *Serratia* sp. MIE2. Upon reaching an ideal density of 0.9 to 1.0 at 600 nm, two milliliters of freshly harvested bacterial cells were pipetted into LPM. Unless otherwise specified during temperature optimization, all infected LPM broths were kept at 27°C for a full 24 hours. The quantity of molybdenum-blue that was produced was determined by pipetting one milliliter of LPM and measuring it at 865 nm [18].

Intelligent issue solver, STATISTICA neural network software from stat soft co., ltd (Tokyo, Japan) with a multilayer feedforward (MLP) and radial basis function (RBF) network as the network was used to assess the experimental findings collected from these tests. Automated neural network analysis included two phases: training and testing. The experimental data was randomly split between the two stages, with half going into training and half into testing. During the training phase, four activation functions were utilized for the hidden and output layers: identity, logistic, tanh, and exponential.

The optimum layer selection was evaluated based on the lowest selection error and highest coefficient of determination (R^2). For optimization and prediction, the top network was chosen. Using the root-mean-square error (RSME) (Eq. 2) and coefficient of determination (R^2) (Eq. 1) between the experimental values and the network's projected values, we assessed the model's training and testing performances. The model's low ADD and RSME values characterize the system's accurate behavior, and its high correlation and determination (R^2) values are indicative of a good model [12].

$$R^2=1-\frac{\sum_{i=1-n}(\text{model prediction}_i-\text{experimental value}_i)^2}{\sum_{i=1-n}(\text{avrage experimental value}-\text{experimental value}_i)^2} \quad \text{(Eqn. 1)}$$

$$RMSE=\sqrt{\frac{\Sigma(y_{i,\exp}-y_{i,cal})^2}{n}} \quad \text{(Eqn. 2)}$$

with p being the number of runs in the experiment and $y_{i,exp}$ and $y_{i,cal}$ being the calculated and experimental responses, respectively. when n is the total amount of data from the experiments.

Preparation of crude Mo-reducing enzyme

The phosphate concentration was modified to 100 mM and *Serratia* sp. MIE2 was cultured in 9 liters of high phosphate medium (HPM). No amount of HPM could induce bacterial production of Mo-blue. The cell still has an active enzyme, even though a high phosphate concentration hinders molybdate reduction to create Mo-blue [14,15]. All experiments were conducted at 4oC unless otherwise indicated to make sure the heat didn't kill the cells. An HPM was centrifuged at 10,000 xg for 20 minutes at 4°C in order to extract the bacterial cells. This was followed by three washes with distilled water for the cells. Following resuspension in Tris-HCl buffer (pH 7.0), the cells underwent a 10-minute re-centrifugation run at 10,000 xg. The pellets combined with 10 ml of 50 mM Tris Buffer (pH 7.0) that contained 2 mM EDTA, 1 mM

PMSF (a protease inhibitor), and 2 mM DTT to make a new solution. Biosonik 111TM sonication was subsequently applied to the cells.

A total of two hours of sonication was completed by subjecting the mixture to one minute of sonication on an ice bath followed by three minutes of cooling. After the samples were sonicated, the pellet cells dissolved and the color turned from light yellow to light pink. The ultra-centrifuged fraction, which included the crude enzyme, was obtained by subjecting the sonicated fraction to a 90-minute centrifugation run at 4°C and 24,000 × g. The experiments were conducted at 4oC with stirring for 10 hours using a 40 to 50% ammonium sulfate fraction dialyzed in 5 L of a 10 mM Tris-HCl pH 7.5 buffer that contained 0.1 mM dithiothreitol. In this study, the proportion of ammonium sulfate had a high activity comparable to that of Shukor et al. [14]. Previously, in Shukor et al. [16], 50 mM of Tris-HCl pH 7.5 buffer containing 0.1 mM beta-mercaptoethanol was substituted with dithiothreitol due to early research showing that beta-mercaptoethanol reduces the activity of Mo-reducing enzymes after long-term storage [14,15].

RESULT

Optimization and prediction of optimal points using artificial neural network (ANN)

Effect of initial pH and temperature on molybdate reduction. Conical flasks were used to incubate the MIE2 strain at various initial pH levels, ranging from 5.5 to 8. According to the data shown in **Fig. 1**, the ideal starting pH was 6.0, while pH 5.5 and 7.5 inhibited decrease. **Fig. 2** shows the temperature effect across a large temperature range (15 to 45°C), with the optimal range being 27 to 35 °C, and no statistically significant difference ($p>0.05$) was found between the data recorded. The synthesis of molybdenum blue from strain MIE2 was inhibited by temperatures higher than 35°C. In order to forecast the best ideal value, the data collected from both effects were processed and fitted with ANN (Table 1). For pH and temperature, the optimal architecture was a network of RBF (1-2-1) nodes. In this RBF (1:2:1), the input, hidden, and output layers each have a specific number of neurons. All neurons from the hidden layer and the output layer neuron have Gaussian and identity as transfer functions for both parameters. The network was chosen due to the R2 values of the training and testing sets being close to 1.0 and showing fewer errors than other networks. The optimum points for pH and temperature predicted by ANN were 6.5 and 30 ºC, respectively. The predicted point gives the highest molybdenum-blue production was chosen as the best point in this predictive analysis

Effect of electron donor and nitrogen sources on molybdate reduction

Fig. 3 shows that among the nitrogen sources and electron donors that were evaluated, 2% sucrose was the most successful in reducing molybdate. The best supply of nitrogen was determined to be ammonium sulfate at a concentration of 1% (**Fig. 4**). When grown in a medium containing sucrose, strain MIE2 produced the largest amount of molybdenum blue. Effectiveness was then found for glucose, fructose, maltose, and galactose in decreasing order.

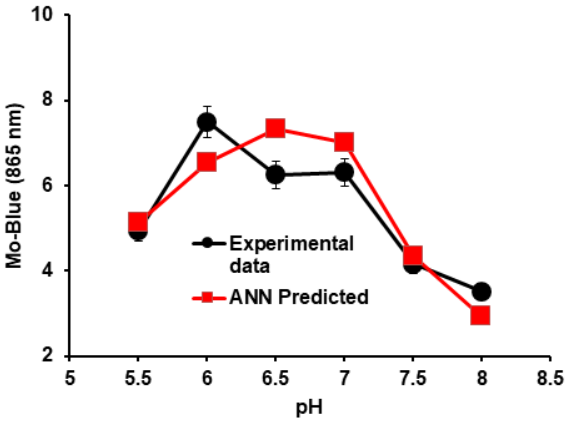

Fig. 1. Influence of starting pH on MIE2 strain molybdenum reduction. Error bars represent mean ± standard deviation (n=3).

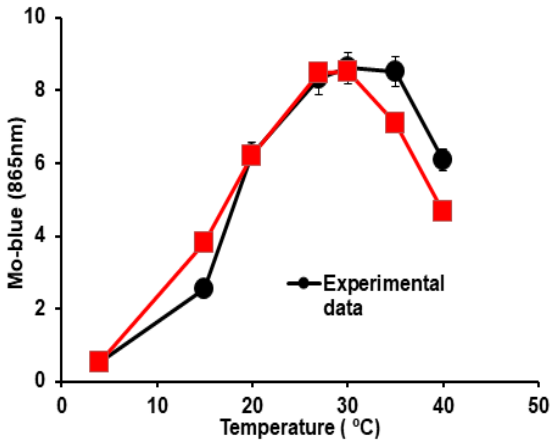

Fig. 2. Study on the temperature-dependent molybdenum reduction by the MIE2 strain. Error bars represent mean ± standard deviation (n=3).

The formation of molybdenum blue was not supported by starch, arabinose, or raffinose. The optimal nitrogen source to facilitate molybdenum reduction was determined to be ammonium sulfate during the screening process. Tryptone, ammonium chloride, glycine, L-alanine,caffeine, urea, and ammonium acetate gave lower molybdenum blue ($p< 0.05$) compared to ammonium sulphate. There was no evidence that glutamic acid or L-glutamine could facilitate molybdenum blue synthesis (data not shown). No statistically significant difference was found among the values of 30–50 g/L of sucrose, as shown in **Fig. 3** ($p>0.05$).

The optimum point of sucrose concentration predicted by ANN was 30 g/L, with MLP (1-3-1) chosen as the network. Regarding transfer functions, every neuron in both the hidden and output layers uses an exponential or identity function. With this MLF (1:3:1), the input, hidden, and output layers each have a certain number of neurons. **Fig. 4** shows that the optimum of ammonium sulphate concentration was 10 g/L. ANN also predicted the same values with experimental with MLP (1-3-1) was chosen as the best network. All hidden and output layers neurons have tanh and exponential as transfer functions.

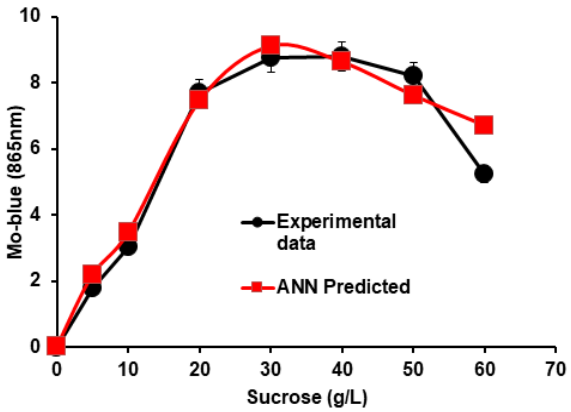

Fig. 3. The impact of various sources of electron donors. A variety of electron donors and 10 mM molybdate were added to low phosphate media in which strain MIE2 was cultured. Error bars represent mean ± standard deviation (n = 3)

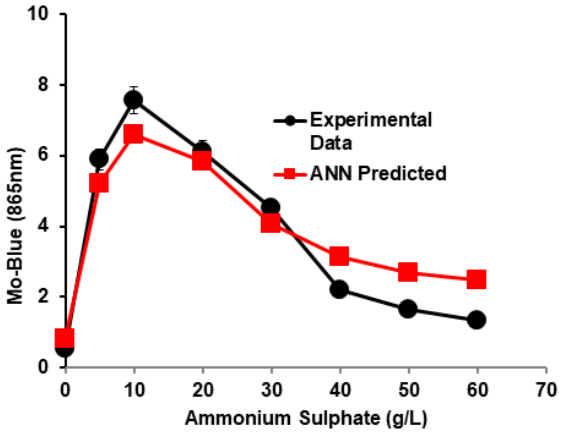

Fig. 4. Different sources of nitrogen and their effects. The MIE2 strain was cultured using sucrose as an electron source in low phosphate environment that also contained 10 mM molybdate. Error bars represent mean ± standard deviation (n = 3).

Effect of phosphate and molybdate concentration on molybdate reduction

The optimum concentration of phosphate concentration was 2 mM (**Fig. 5**). The concentration over than 2 mM was inhibitory to molybdenum blue production. The best optimum point predicted by ANN for phosphate concentration was 5 mM with MLP (1-4-1) was chosen as the best network. All neurons from the hidden layer and the output layer neuron have tanh as transfer function parameters. Otherwise, the optimum concentration of molybdate occurred at 10 mM (**Fig. 6**). The optimum values predicted by ANN occurred at between 10-20 mM with 20 mM was chosen as the best predicted value in this analysis with MLP (1-3-1) was chosen as the best network. All neurons from the hidden layer and the output layer neuron have exponential and logistic as transfer functions.

Validation experiment

The validation compared the experimental optimal point with the ANN-predicted optimal point for Mo-blue production by *Serratia* sp. MIE2. Validation experiments followed the optimal point outlined in **Table 2**. The validation of the ANN-predicted optimal point showed that Mo-blue production increased to an absorbance of 14.0 at 865 nm, compared to the experimental optimal point, which only reached an absorbance of 9.7.

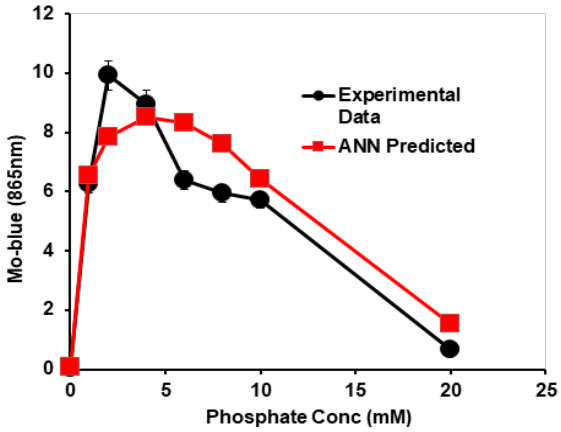

Fig. 5. The MIE2 strain's molybdenum reduction as a function of phosphate concentration. Error bars represent mean ± standard deviation (n = 3).

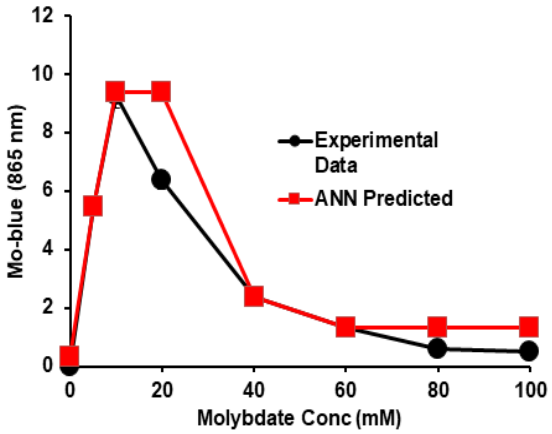

Fig. 6. The MIE2 strain's molybdenum reduction as a function of molybdate concentration. Error bars represent mean ± standard deviation (n = 3).

Table 1. Summary of active networks of each variable.

Parameters	Net. name	Training perf.	Test perf.	Training error	Test error	Training algorithm	Hidden activation	Output activation	R^2	RSME
pH	RBF 1-2-1	0.9995	0.9555	0.0001	0.010807	RBFT	Gaussian	Identity	0.8059	0.031963
Temperature	RBF 1-2-1	0.9995	0.9555	0.0001	0.010808	RBFT	Gaussian	Identity	0.9377	0.013172
Sucrose conc	MLP 1-3-1	0.9637	0.9000	0.0129	0.115727	BFGS 6	Exponential	Identity	0.96	0.003924
Ammonium Sulphate conc	MLP 1-3-1	0.9637	0.9000	0.0129	0.115727	BFGS 6	Tanh	Exponential	0.956	0.035422
Molybdate conc	MLP 1-3-1	1.0000	0.9982	0.0000	0.054669	BFGS 6017	Exponential	Logistic	0.9226	0.039000
Phosphate conc	MLP 1-4-1	0.9504	1.0000	0.0088	0.014971	BFGS 7	Tanh	Tanh	0.8326	0.006663

Table 2. Experimental and ANN predicted value for each variable.

	pH	Temperature (ºC)	Sucrose conc (g/L)	Ammonium sulphate conc (g/L)	Molybdate conc (mM)	Phosphate conc (mM)	Validation (Mo-Blue-865 nm)
Experimental	6.0	27	30	5	10	2	9.7
ANN	6.5-7.0	27-35	30-40	5-10	10-20	4-6	14

A study on the molybdenum reduction effect of solvents and insecticides

The effects of several pesticides on bacterial cells and molybdenum-reducing enzyme activity are shown in **Fig. 7**. A comparison was made between the control and bacterial cells exposed to carbofuran, diazinon, methomyl, malathion, trichlorfon, bendiocarb, and carbaryl at 1 ppm, and the results showed inhibition rates below 50%. In contrast, no inhibitory effects were observed for propoxur, parathion, dimethoate, chlorpyrifos, atrazine, or simazine. Diazinon, bendiocarb, carbaryl, chlorpyrifos, atrazine, and simazine were the only pesticides that did not reduce the molybdenum-reducing enzyme's activity, whereas the majority of the others affected it by less than 30%. Only hexane and butanol, as shown in **Fig. 8**, were able to limit bacterial cell molybdenum blue synthesis by more than 20%. When contrasted with the control, other solvents showed inhibition levels below 20%. The inhibition of the molybdenum-reducing enzyme was less than 20% in all solvents tested, suggesting that it had little effect even at a 10% concentration.

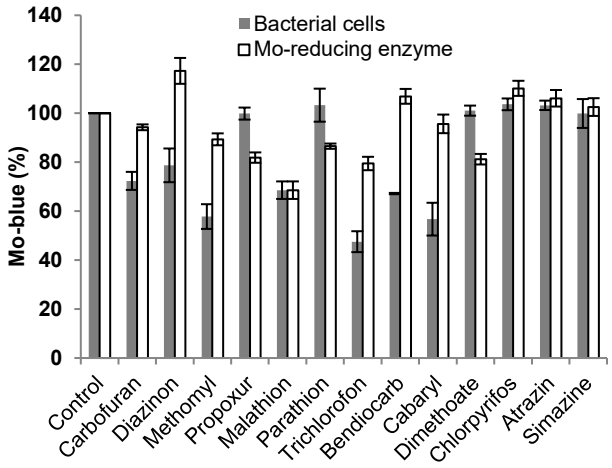

Fig. 7. The Mo-reducing enzyme and bacterial cell viability as a result of pesticide exposure. The error bars represent mean ± standard deviation for three replicates.

DISCUSSION

In this work, we have isolated a new Mo-reducing bacterium from agricultural soil for the first time. According to previous works, most Mo-reducing bacteria were isolated from the polluted soil collected from galvanic factories and workshops. Soil microbes, for example, may not be able to handle pollution in different environments as well as those in agricultural soil. Commercial bacteria or other degraders from other regions aren't always the best option; sometimes, autochthonous microbes—isolated from particular polluted soils, expanded to a huge scale, and then returned to the contaminated soils—work better. The rising number of reports of new microbial strains that can break down certain xenobiotics or heavy metals is a prime example of this trend [17–23].

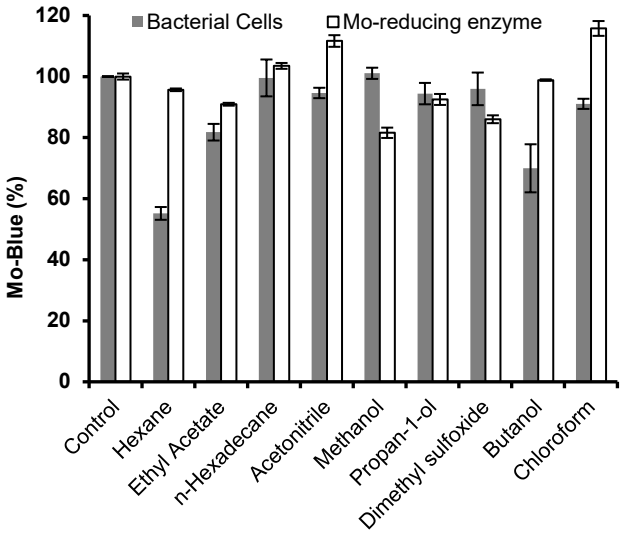

Fig. 8. Solvent effects on bacterial cell membranes and molybdenum-reducing enzyme activity. The error bars represent mean ± standard deviation for three replicates.

When reducing molybdenum, temperature and pH are crucial factors. Both characteristics have the potential to influence protein folding and enzyme activity, which in turn inhibits molybdenum reduction because this process is enzyme-mediated. Malaysia, a tropical country with an average annual temperature range of 25 to 35 °C, would benefit from bioremediation at an ideal temperature for a wide range of 27 to 35 °C [24]. Therefore, strain MIE could be a candidate for soil bioremediation of molybdenum locally and in other tropical countries. Strain MIE2 reduces molybdenum optimally at pH 6, indicating that this bacterium is a neutrophile bacterium that is able to grow between pH 5.5 and 8.0. During the growth of strain MIE2 in low phosphate media, the pH of the media dramatically decreased from pH 6 to 5 before molybdenum reduction took place. Since acidic pH plays an essential role in forming phosphomolybdate before it is reduced to molybdenum blue, this indicates that the species could also form during growth on molybdenum.

Previous works by Shukor's lab [15,25–27] demonstrated that all of the Mo-reducing bacteria from the genus *Serratia* use sucrose as the best carbon source. However, molybdenum reduction in *E.* coli K12 [28] and *E. cloacae* strain 48 prefer glucose as a better carbon source than sucrose [12]. Bacteria can produce electron-donating substrates like NADH and NADPH when carbon sources are present in the medium by means of metabolic processes like glycolysis, the Krebs cycle, and the electron transport chain. The molybdenum-reducing enzyme relies on NADH and NADPH substrates to facilitate reduction by providing electrons.

The production of nucleic acids, enzymes, and amino acids—essential building blocks for bacterial metabolism and growth—requires a nitrogen source. When we grew strain MIE2 with ammonium sulfate as the nitrogen source, we saw the greatest reduction of molybdate. Consistent with earlier studies on molybdenum reduction, this confirms that ammonium sulfate is

the nitrogen source of choice for numerous Serratia species, renowned for their molybdenum-reducing abilities. The molybdenum reduction process relies on proteins and enzymes, and this preference probably helps with that [15,25–27].

Knowing the ideal amounts of phosphate and molybdate to facilitate molybdenum reduction is crucial, as these anions limit bacterial molybdenum blue synthesis. Strain MIE2 needed 2 mM phosphate for optimum reduction, whereas other Mo-reducing bacteria have been found to require 5 mM of phosphate [29–31]. Research on the effects of different molybdenum concentrations revealed that strain MIE2 could decrease molybdenum concentrations up to 60 mM. However, this was accompanied by a decrease in Mo-blue synthesis. This strain could mitigate the high levels of molybdenum contamination by reducing the concentration to insoluble.

A neural network (ANN) is a type of computational method that mimics the way the brain processes data. Like the human brain, it learns from its environment and uses the strength of connections between interneurons, also called synaptic weights, to store the information it has learned. The network is composed of widely dispersed adaptive nonlinear processing elements, or neurons. Because of these ANN features, data fitting, prediction, and the modeling of nonlinear relationships are all made easier and more flexible [31]. Here, we showcased ANN's capacity to forecast the optimal point by fitting optimization experimental data. The findings demonstrate that ANN can accurately fit experimental data, as evidenced by an R2 close to one and an RMSE approaching zero. The best concentrations of pH (6.5), temperature (30 ºC), ammonium sulphate (30 g/L), molybdate (20 mM), and phosphate (4 mM) were predicted by artificial neural networks (ANN) based on the predictive analysis. Verifying the ANN-predicted point revealed a rise in Mo-blue production from 9.7 to 14.1 nm at 865 nm (**Table 2**).

In molybdenum reduction, the concentrations of molybdate and phosphate are critical because an excessive amount of phosphate can destabilize the intermediate molybdate and inhibit the synthesis of molybdenum blue. Therefore, for the maximum Mo-blue generation, the phosphate to molybdate proportion is crucial [29–31]. It may be inferred from the maximum Mo-blue production following predictive analysis that ANN can accurately predict the ratio of the two parameters. Therefore, the study concluded that the prediction analysis was successful, and it shown that ANN can be a robust fitting method.

Since pesticides are commonly found in agricultural settings, they are considered persistent organic pollutants. Severe ecological consequences and effects on bioremediation could result from pesticides that disrupt the activity of soil microbes, altering soil nutritional quality [32]. In order to determine how well strain MIE2 reduced molybdate in the presence of pesticides and Mo-reducing enzymes, experiments were conducted to study the effects of these inhibitors on bacterial cells.

The results indicate that pesticides impede the metabolic process of strain MIE2, reducing Mo-blue formation, since they function as respiratory inhibitors. since a result, bacterial cells exhibit less inhibition compared to Mo-reducing enzyme. Because of its use as a pesticide solvent, the study assessed the effects of solvents. Bacterial cells and the Mo-reducing enzyme were relatively unaffected by the presence of solvents, suggesting that strain MIE2 has solvent tolerance and can function in various solvent environments.

CONCLUSION

Finally, the first molybdenum-reducing bacterium was isolated from agricultural soil, and this was reported. The dialysis tubing experiment clearly shows that enzymatic reduction is the primary mechanism for reducing molybdenum to Mo-blue. With an increase in Mo-blue production from 9.7 to 14.0 absorbance nm at 865 nm, the point predicted by ANN was confirmed. The highest Mo-blue production after predictive analysis implies that ANN can predict the best point for mo-blue maximum output. Thus, the predictive analysis was considered successful, and the capability of ANN to be a robust fitting tool was proven in this study. Pesticide and solvent effects demonstrated a 50% inhibition of bacterial cell viability. On the other hand, the Mo-reducing enzyme was unaffected by either inhibitor. Using multivariate analysis and response surface methodology (RSM), we are currently optimizing this bacterium and purifying its Mo-reducing enzyme to homogeneity.

ACKNOWLEDGMENT

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