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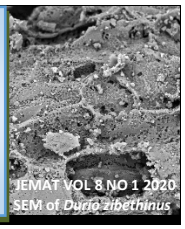


# Kinetic Modelling of Molybdenum-blue Production by *Bacillus* sp. strain Neni-10

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**ABSTRACT**

Kinetic modelling of bacterial reduction process reveals vital parameters like specific reduction rate, hypothetical maximum reduction and deduce whether high substrate (molybdenum) concentration affects the lag phase of the reduction. The commonly used natural logarithmic transformation to linearize the reduction process seems inaccurate as it gives only an approximate value for the specific growth rate. This work for the first time utilized eight different primary models such as Gompertz, Baranyi-Roberts, Logistic, Von Bertalanffy, Richards, Schnute, Buchanan three-phase Huang to obtain values of the kinetic constants that could further be used for secondary modelling. Baranyi-Roberts model was the best model fitting Mo-blue production curve in *Bacillus* sp. strain Neni-10 based on statistical values for RMSE (root-mean-square error), $R^2$ (adjusted coefficient of determination), AICc (corrected Akaike Information Criterion) BF (bias factor) and AF (accuracy factor). The fitting parameters obtained were lag time ($\lambda$), maximal Mo-blue production ($Y_{max}$) and maximum Mo-blue production rate ($\mu_m$). The use of microbial growth models to get accurate Mo-blue production rate is entirely new to molybdenum reduction (detoxification) process and the kinetic constants obtained could be very useful secondary modelling. This work has revealed the usefulness of these models in modelling bacterial Mo-blue production.

## INTRODUCTION

Bacterial growth-associated processes often exhibit a unique phase where specific growth rate begins at zero value producing a lag time ($\lambda$), after which it accelerates to a maximal value ($\mu_{max}$) for a certain time. The sigmoid-shaped lag period has been argued to be due to the bacterial cells adjusting to the new environment and gearing their growth mechanism in a vegetative phase particularly during storage. The adjustment period (lag period) has been suggested as a transient period that links two autonomous systems. The introduction of the lag time or parameter is mainly for convenience rather than having a mechanistic interpretation [1]. It was hypothesized that, each bacterial cell in the initial innocula, would have different growth rates, that could show nonlinear distribution as earlier suggested [1,2].

Molybdenum as ubiquitous heavy metal has numerous industrial uses including as corrosion resistant steel, lubricant, alloying agent, anti-freeze in automobile engine and as molybdenum disulphide. These industrial wide spread applications of molybdenum have resulted in pollution of several water bodies worldwide as earlier reported in Tyrol in Austria, Tokyo Bay in Japan and the Black Sea, where hundreds of ppm molybdenum level were found [3]. Additionally, significant sewage sludge pollution that poses a health hazard was terrestrially reported [3]. Molybdenum in elemental form or in different chemical combination was reported to be toxic to ruminants particularly cow at concentrations as low as parts per million (ppm) [4,5]. The perceived lower human molybdenum toxicity case compared to other heavy metals like chromium, mercury, and cadmium is attributed to less attention been paid and has resulted in little works on molybdenum detoxification (bioreduction) process. To date, a number of molybdenum-reducing bacteria have been

locally isolated [6–13] with few exceptional commercial strains [14–17]. However, with recent data on molybdenum toxicity by inhibiting spermatogenesis and arresting embryogenesis in catfish and mice at levels as low as ppm [18,19] calls for more works on microbial molybdenum detoxification.

The kinetic studies on microbial Mo-blue production have been explored [20,21], however, these works linearize the Mo-blue production over time profile to obtain the specific growth rate for further secondary modelling. Perhaps the benefits of nonlinear regression analysis for the Mo-blue production have been described. Therefore, this work focus on evaluating the available models such as Gompertz [22,23], Logistic [23,24], Baranyi-Roberts [25], Richards [23,26], Schnute [23], Buchanan three-phase [27], Von Bertalanffy [28,29] and the recently Huang model [30] (**Table 1**) to model Mo-blue production by *Bacillus* sp. strain Neni-10.

**Table 1**. Models used to model Mo-blue production *Bacillus* sp. strain Neni-10.

| Model | p | Equation |
|---|---|---|
| Modified Logistic | 3 | $y = \frac{A}{\left\{1+\exp\left[\frac{4\mu_m}{A}(\lambda - t)+2\right]\right\}}$ |
| Modified Gompertz | 3 | $y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t)+1\right]\right\}$ |
| Modified Richards | 4 | $y = A\left\{1+v\exp(1+v)\exp\left[\frac{\mu_m}{A}(1+v)\left(1+\frac{1}{v}\right)(\lambda - t\right.\right.$ |
| Modified Schnute | 4 | $y = \left(\mu_m \frac{(1-\beta)}{\alpha}\right)\left[\frac{1-\beta\exp(\alpha\lambda+1-\beta-\alpha t)}{1-\beta}\right]^{\frac{1}{\beta}}$ |
| Baranyi-Roberts | 4 | $y = A+\mu_m x+\frac{1}{\mu_m}\ln\left(e^{-\mu_m x}+e^{-h0}-e^{-\mu_m x-h_o}\right)$ $-\ln\left(1+\frac{e^{\mu_m x+\frac{1}{\mu_m}\ln\left(e^{-\mu_m x}+e^{-h0}-e^{-\mu_m x-h0}\right)}-}{e^{\left(y_{\max}-A\right)}}\right.$ |
| Von Bertalanffy | 3 | $y=K\left[1-\left[1-\left(\frac{A}{K}\right)^3\right]\exp\left(-\left(\mu_m x/3K^{\frac{1}{3}}\right)\right)\right]^3$ |
| Huang | 4 | $y = A+y_{\max}-\ln\left(e^{A}+\left(e^{Y_{\max}}-e^{A}\right)e^{-\mu_m B(x)}\right)$ $B(x)=x+\frac{1}{\alpha}\ln\frac{1+e^{-\alpha(x-\lambda)}}{1+e^{\alpha\lambda}}$ |
| Buchanan Three-phase linear model | 3 | Y = A, IF X < LAG<br>Y=A + K(X−λ), IF λ < X > |

**Note:**
A= Mo-blue lower asymptote;
$\mu_m$= maximum specific Mo-blue production rate;
v= affects near which asymptote maximum Mo-blue production occurs.
λ=lag time
$y_{max}$= Mo-blue upper asymptote;
e = exponent (2.718281828)
t = sampling time
α,β, k = curve fitting parameters
$h_0$ = a dimensionless parameter quantifying the initial physiological state of the reduction process. The lag time ($h^{-1}$) can be calculated as $h_0=\mu_{max}$

## MATERIALS AND METHODS

### Maintenance of the Molybdenum-reducing bacterium

The bacterium previously isolated, identified and characterized by Mansur *et al.* [11] was maintained on low phosphate agar media (pH 7.0) containing glucose (1%), $Na_2MoO_4.2H_2O$ (0.242%), $Na_2HPO_4$ (0.071% or 5 mM), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%) and yeast extract (0.0.5%) [9]. Though, glucose was separately autoclaved.

### Preparation of resting cells for molybdenum reduction characterization

Static culture using resting cells in a microplate was used to Monitor the Mo-blue production at various sodium molybdate concentration [31]. Cells grown in 1 L high phosphomolybdate media (HPM=100 mM phosphate) on orbital shaker (150 rpm) at room temperature overnight were harvested by centrifuging at 12,000 x g for 10 minutes. The pellet was then rinsed three times with 20 ml of low phosphate media (LPM) devoid molybdenum to remove suspended cells and residual phosphate to an absorbance of approximately 1.00 at 600 nm. The higher concentration (100 mM) was found to strongly inhibit Mo-blue production [6,21,32–38]. Sterically 180 µL of various concentrations of sodium molybdate dispensed into each well of a sterile microplate, followed by the inoculation with 20 µL from a stock culture to initiate Mo-blue production. A sterile sealing tape that allows gas exchange (Corning® microplate) was used to seal the plate and incubated at room temperature. Periodically, absorbance at 750 nm was read using BioRad microtiter plate reader (Richmond, CA, Model No. 680) to monitor the Mo-blue production from the media using a specific extinction coefficient of 11.69 $mM^{-1}.cm^{-1}$ [39].

### Determining the kinetic parameters of Mo-blue production

### Data fitting

CurveExpert Professional software (Version 1.6) was used to conduct nonlinear regression to fit-in the growth data involving the Marquardt algorithm that minimizes sums of square of residuals. This lookup approach minimizes the sum of squares of the differences between the observed and predicted values. Input into the software can be manually or automatically programmed to calculate initial values of parameters. The estimation of $\mu_m$ involves the steepest ascent search of the curve among four datum points. While λ estimation was conducted by determining the intersection of this line with x axis. Estimation of asymptote ($A$) was finally done by taking the final datum point.

**Statistical analysis**

To ascertain if there is significant difference between models with different number of parameters, with regards to the fitness quality of same experimental data, statistics like Root-Mean-Square Error (RMSE), adjusted coefficient of determination ($R^2$), corrected AICc (Akaike Information Criterion), bias factor (BF) and accuracy factor (AF) could be used.

The RMSE was calculated according to **Eqn. 1**, where $n$ is the number of experimental data, $p$ is the number of parameters assessed by the model, $Ob_i$ is the experimental data and $Pd_i$ are the values predicted by the model. It is predicted that a model with smaller number of parameters will give a smaller RMSE values [40].

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(Pd_i - Ob_i)^2}{n-p}} \quad \textbf{(Eqn. 1)}$$

The quality of fit of a model in a linear regression is assessed using $R^2$ (coefficient of determination). However, $R^2$ does not provide comparable analysis in a nonlinear regression where the number of parameters between the models vary, as such adjusted $R^2$ is used to calculate the quality of the nonlinear models employing equations **2** and **3**. According to the formula, $S_y^2$ is the total variance of the y-variable and RMS is Residual Mean Square.

$$Adjusted\left(R^2\right) = 1 - \frac{RMS}{s_Y^2} \quad \textbf{(Eqn. 2)}$$

$$Adjusted\left(R^2\right) = 1 - \frac{\left(1-R^2\right)(n-1)}{(n-p-1)} \quad \textbf{(Eqn. 3)}$$

The Akaike Information Criterion (AIC) provides solution to the model selection by way of computing the relative quality of a given statistical model in given set of experimental data [41]. However, corrected AIC or AICc is used for data set with high number of parameter or having a smaller number of values [42]. For each data set in a model, the AICc is calculated based on the following equation (**Eqn. 4**);

$$AICc = 2p + n\ln\left(\frac{RSS}{n}\right) + 2(p+1) + \frac{2(p+1)(p+2)}{n-p-2} \quad \textbf{(Eqn. 4)}$$

Where $n$ and $p$ represent number of data points in the curve and the number of parameters utilized in the model, respectively. This procedure considers variations in the number of parameters and the goodness-of-fit between two models. Perhaps, for every set of data, the model with smallest AICc value is more likely correct [42].

The Bias and Accuracy factors (BF and AF) were calculated according to Eqns. **5** and **6** to test the goodness-of-fit of the models [43]. The value of the Accuracy Factor is usually ≥ 1, higher values signifies less precise prediction. In a bacterial growth curves or Mo-blue production, an ideal match between observed and predicted values is achieved when the Bias Factor is equal to 1. A bias factor greater than 1 signifies fail-safe model, whereas, a bias factor less than 1 signifies a fail-negative model.

$$\text{Bias factor} = 10^{\left(\sum_{i=1}^{n}\log\frac{(Pd_i/Ob_i)}{n}\right)} \quad \textbf{(Eqn. 5)}$$

$$\text{Accuracy factor} = 10^{\left(\sum_{i=1}^{n}\log\frac{|(Pd_i/Ob_i)|}{n}\right)} \quad \textbf{(Eqn. 6)}$$

## RESULTS AND DISCUSSION

In this bacterium, the Mo-blue production profile over time gives a sigmoid-shaped with a lag phase of about 15 h. Maximum Mo-blue production occurred at around 50 h of static incubation (**Fig. 1**). Mo-blue production over time was modelled using eight different models, and all the fitting were visually acceptable (**Fig. 2**). Statistical analysis reveals Baranyi-Roberts model as the best fitting with lowest AICc and RMSE values, and highest adjusted $R^2$value. The AF and BF values were equally excellent for the model with values closest to 1.0 (**Table 2**). The coefficients for the Baranyi-Roberts model at various molybdenum concentrations are shown in **Table 3**.

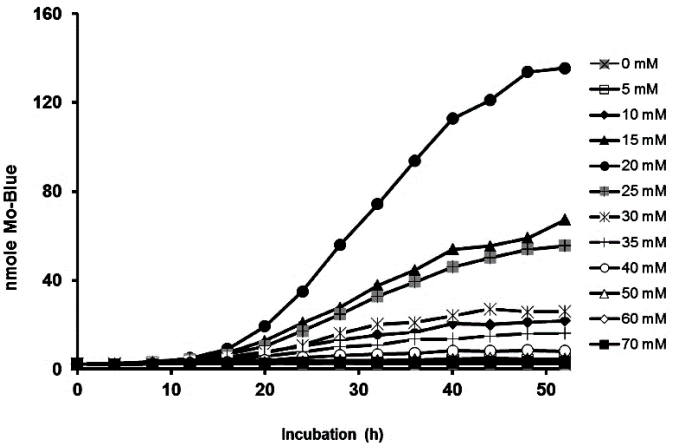


**Fig. 1.** Mo-blue production curves of *Bacillus* sp. strain Neni-10 at various sodium molybdate concentrations over time. Data represented as mean ± SD of three replicates.

**Table 2.** Statistical analysis for various fitting models.

| Model | p | RMSE | $R^2$ | $adR^2$ | AICc | BF | AF |
|---|---|---|---|---|---|---|---|
| Huang | 4 | 0.048 | 0.999 | 0.999 | -64.15 | 1.11 | 1.24 |
| Baranyi-Roberts | 4 | 0.128 | 0.994 | 0.991 | -36.66 | 1.20 | 1.27 |
| Buchanan-3-phase | 3 | 0.020 | 0.994 | 0.993 | -94.76 | 1.01 | 1.09 |
| modified Logistics | 3 | 0.089 | 0.997 | 0.996 | -52.55 | 0.99 | 1.20 |
| modified Richards | 4 | 0.022 | 0.992 | 0.990 | -86.42 | 0.82 | 1.24 |
| von Bertalanffy | 3 | 0.101 | 0.996 | 0.994 | -49.04 | 0.58 | 1.88 |
| modified Gompertz | 3 | 0.022 | 0.997 | 0.999 | -99.79 | 1.01 | 1.01 |
| modified Schnute | 4 | 0.089 | 0.997 | 0.995 | -47.00 | 1.24 | 1.26 |

**Note**:
*p* no of parameters
$adR^2$ Adjusted Coefficient of determination
BF Bias factor
AF Accuracy factor

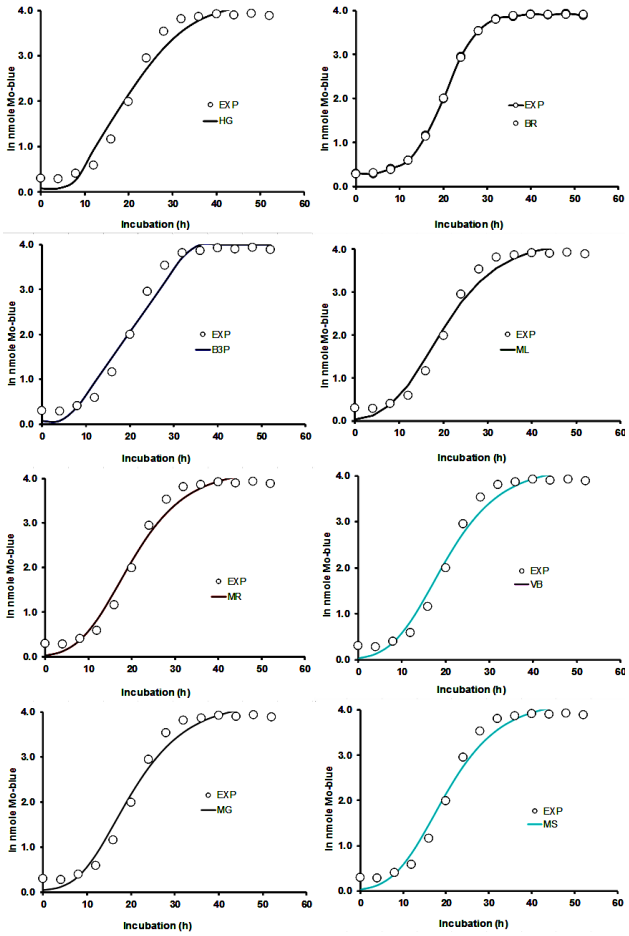


**Fig. 2.** Mo-blue production curve of *Bacillus* sp. strain Neni-10 at 20 mM sodium molybdate fitting various models such as Huang (HG), Baranyi-Roberts (BR), Buchanan-three phase (B3P), modified Logistics (ML), modified Richards (MR), von Bertalanffy (VB), modified Gompertz (MG) and modified Schnute (MS).

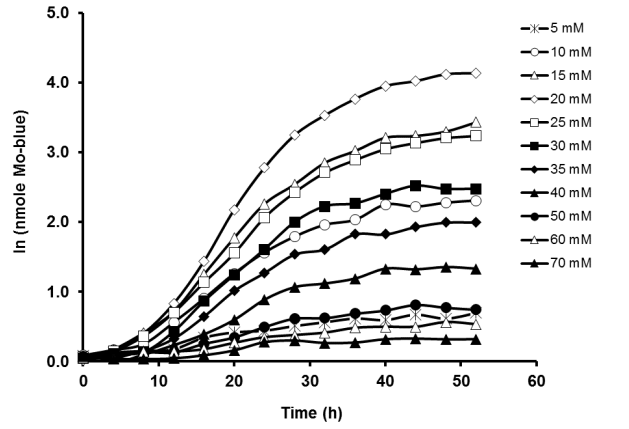


**Fig. 3.** Mo-blue production curves of *Bacillus* sp. strain Neni-10 on various sodium molybdate concentrations fitted Baranyi-Roberts model.

**Table 3**. Mo-blue production coefficients at various molybdenum concentrations modelled using Baranyi-Roberts model.

| | Molybdenum concentration (mM) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 50 | 60 | 70 |
| Asymptote (ln nmol Mo-blue) | 0.45 | 1.29 | 1.80 | 2.59 | 3.54 | 3.69 | 3.14 | 1.71 | 1.47 | 0.86 | 0.50 |
| $\mu_m$ ($h^{-1}$) | 0.02 | 0.06 | 0.10 | 0.12 | 0.14 | 0.15 | 0.14 | 0.13 | 0.08 | 0.06 | 0.04 |
| lag (h) | 6.92 | 11.37 | 12.36 | 12.13 | 12.07 | 12.45 | 12.89 | 13.86 | 13.56 | 13.44 | 15.07 |

The Baranyi Roberts model fitted the data best by having the highest adjusted $R^2$ value, lowest RMSE and AICc values and the closest values to unity for both Accuracy and Bias factors. This model proposed that **Eqn. 7** (first-order differential equation) describes the variation in the cell population (x) with time [44];

$$\frac{dx}{dt} = \alpha(t)\mu(x)x \qquad \textbf{(Eqn. 7)}$$

Below is the relationship is assumed for growth or production rate (**Eqn. 8**)

$$\mu = \mu_{\max}\left(1 - \frac{x}{x_{\max}}\right) \qquad \textbf{(Eqn. 8)}$$

The generic form of the model can be rewritten as **Eqn. 9.**

$$\mu(t) = \frac{1}{x(t)}\frac{dx}{dt} = \mu_{\max}\alpha(t)f(t) \qquad \textbf{(Eqn. 9)}$$

In the model, $\alpha(t)$ function assumes that growth during the lag phase was inhibited by a 'bottle-neck' intracellular component represented by $P(t)$ in a similar manner to the Michaelis–Menten kinetics. The quotient $q_0$ represented the physiological state of the inoculum, if the ratio between the substance $P(t)$ and its Michaelis–Menten constant grows exponentially, from an initial value $q_0$, at a constant specific rate. The $\alpha(t)$ increases monotonously with the limits $0 \leq \alpha \leq 1$ and $\lim_{t\to\infty} \alpha(t)=1$ as follows (**Eqn. 10**);

$$\alpha(t) = \frac{P(t)}{P(t)+K_p} = \frac{q(t)}{1+q(t)} = \frac{q_0}{q_0 + e^{-\mu_{\max} t}} \qquad \textbf{(Eqn. 10)}$$

The end-of product formation or end-of-growth inhibition is represented by the $f(t)$ function (**Eqn. 11**), which decreases monotonically with $f(0) = 1$ and $\lim_{t\to\infty} f(t) = 0$. The $f(t)$ function is described by a logistic inhibition function in most dynamics models as follows;

$$f(t) = 1 - \left(\frac{x}{x_{\max}}\right) \qquad \textbf{(Eqn. 11)}$$

Solutions to this differential equation was successfully worked out at a certain fixed condition, e.g. isothermal temperatures. The consequence of the solution is for it to have six parameters (**Eqn 12**) (1);

$$y = A + \mu_{\max} x + \frac{1}{\mu_{\max}} \ln\left(e^{-vx} + e^{-h_0} - e^{-vx-h_o}\right) - \frac{1}{m} \ln\left(1 + \frac{e^{m\mu_{\max} x + \frac{1}{\mu_{\max}} \ln\left(e^{-vx} + e^{-h_0} - e^{-vx-h_0}\right)} - 1}{e^{m(y_{\max} - A)}}\right)$$

Where;
$A$ signifies initial concentration of cell (or product concentration), $y_{max}$ is the asymptomatic cell concentration (or product concentration) in ln (CFU/ml) or ln product concentration, $m$ is the curvature parameter, symbolizes the transition from the exponential phase. The $h_o$ is a dimensionless parameter indicating the initial physiological state of the cells, while $v$ is the curvature parameter that characterize the transition to the exponential phase. The maximum specific growth rate ($1/h$) is represented as $\mu_{max}$ or $\mu_m$ and the lag time $\lambda(h)$ equals $h_o/\mu_{max}$.

The parameters for the curvature are; $v = \mu_{max}$ or $\mu_m$ and $m$=1. Decrease in the number of parameters by two results in the model having four parameters left; $\mu_{max}$; $h_0$; $A$ and $y_{max}$ (**Eqn. 13**). Baranyi and Roberts suggested that $h_0$ can be considered as suitability indicator of the microbes to the true environment [1]. If the experimental method is standardized, the suitability indicator may be more or less constant, which can tally with the assumption that λ (lag time) and $\mu_{max}$ (maximum specific growth rate) are inversely proportional.

$$y = A + \mu_{\max} x + \frac{1}{\mu_{\max}} \ln\left(e^{-\mu_{\max} x} + e^{-h_0} - e^{-\mu_{\max} x - h_o}\right) - \ln\left(1 + \frac{e^{\mu_{\max} x + \frac{1}{\mu_{\max}} \ln\left(e^{-\mu_{\max} x} + e^{-h_0} - e^{-\mu_{\max} x - h_0}\right)} - 1}{e^{(y_{\max} - A)}}\right) \quad \textbf{(Eqn. 13)}$$

The mechanistic in attributes of the Baranyi-Roberts model was suggested to be a lot better than the modified Gompertz model, with its parameters having more biological meaning than the modified Gompertz model, in spite the fact that the model has 4 fitting parameters. The Baranyi-Roberts model was selected in fitting the growth profile of *Bacillus* sp. strain Neni-10 based on its mechanistically-inclined properties compared to modified Gompertz model (**Fig. 4**). It was suggested that to increase the statistical significance of a mechanistic model with 4 parameters over non-mechanistic 3-parameter model, the number of data sets obtained be raised [23].

Baranyi and Roberts model has been used successfully to model bacterial growth curves of *Brochothrix thermosphacta*, *Bacillus* spp., *Escherichia coli* O157:H7, *Clostridium* spp., *Listeria monocytogenes*, *Staphylococcus* spp., *Salmonella* Typhimurium and *Yersinia enterocolitica* [1,25,29,45,46]. This model is most preferred due to its excellent fitting capability, the model is suitable for dynamic environmental conditions, and majority of the model parameters have biological meaning [29,47]. Additionally, the Baranyi-Roberts model was used to successfully to model algal growth [48,49].

The biological meaningful coefficients gotten from the fitting process are; lag time (λ), maximal Mo-blue production ($Y_{max}$) and maximum Mo-blue production rate ($\mu_m$), which could be used for the secondary modelling of Mo-blue production using models such as two-parameter Monod or more complex secondary models like Aiba, Yano Haldane, etc. These mechanistic models are often used in basic research with the aim of gaining better understanding of the physical, chemical and biological processes that lead to the growth profile observed. All things being equal, mechanistic models are powerful tools as they tell more about the driving patterns of the underlying processes. They are more likely to work correctly when extrapolating beyond the observed conditions [50].

## CONCLUSION

The Mo-blue production in *Bacillus* sp. strain Neni-10 has been successfully modelled using eight different models, Baranyi-Roberts model was the best model fitting the Mo-blue production curve based on statistical values for RMSE (root-mean-square error), ad$R^2$ (adjusted coefficient of determination), AICc (corrected Akaike Information Criterion), AF (accuracy factor) and BF (bias factor). Parameters obtained from the fitting exercise were maximum Mo-blue production rate ($\mu_m$), lag time (λ) and maximal Mo-blue production ($Y_{max}$) of X ($h^{-1}$), Y (h) and Z (nmol Mo-blue), respectively. In heavy metals detoxification process by microbes, the use of growth curve models to get accurate Mo-blue production rate that could be utilized further for secondary modelling is novel as demonstrated by literature search. This work has therefore established the applicability of such models. Work is still ongoing to conduct secondary modelling on the inhibitory effects of substrate (molybdenum), pH and temperature on maximum Mo-blue production rate.

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