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Bacillus pasteurii mediated ureolysis – a numerical approach for kinetic analysis

Ureolysis kinetics in *Bacillus pasteurii*

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***Abstract*— Microbiological hydrolysis of urea (ureolysis) is a natural geochemical process which is closely linked with carbonate precipitation, or biocalcification. However, for sake of simplicity, ureolysis rate has often been considered to be first order with respect to urea concentration and quantitative role of microorganism remains underestimated. In this study, ureolysis and growth of ureolytic microorganism *Bacillus pasteurii* has been closely investigated in laboratory batch experiments at three different levels of initial urea and biomass concentration. Results suggest though biomass growth to be independent of initial urea concentration but ureolysis depends on both of the parameters. Experimental data modelling with a number independent rate expressions for biomass growth (*i.e.* exponential, logistic, Gompretz, modified logistic), ureolysis (1st order, pseudo-first order) as well as coupled (Monod, Contois model), reveals a modified logistic expression for growth and Michaelis–Menten substrate utilization kinetics for ureolysis were the most suitable representation.**

***Kewwords*— ureolysis, modelling, modified logistic, regression**

I. Introduction

Microbiologically driven calcium carbonate precipitation (referred as biocalcification) is an environmentally ubiquitous biogeochemical process and has been extended in large number of environmental engineering applications ranging from solid-phase contaminant capture [1], soil improvement [2], strengthening concrete structures [3], [4], wastewaters treatment [5], carbon sequestration [6], [7]. Fundamentally the process is driven through heterotrophic microbial urea hydrolysis which simultaneously increases dissolved inorganic carbon and creates an alkaline microenvironment shifting carbonate–bicarbonate equilibrium toward carbonate, resulting in a state of oversaturation and subsequent precipitation of $CaCO_3$ [6], [8], [9].

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Urea is an important organic nitrogen carrier in natural environments and production of urease allows bacteria to make use of urea as a nitrogen source. The ability to hydrolyse urea is widely distributed among indigenous bacteria present in soils and groundwater environments [10]. Kinetics of calcium carbonate precipitation in response to the hydrolysis of urea has extensively been investigated [11], [12]. However, in nearly all proposed models the very first ureolysis step has been over-simplified to be first order with respect to urea concentration, neglecting any microbial participation [11], [13]–[15]. Paradoxically in different studies (including some of those referred above), authors have also reported the process performance to be dependent on urea content as well as inoculum size [13], [14], [16]. In addition, the estimated rate constant across different studies varied widely (between 0.05 and 0.9 d^{-1}) – which clearly elucidate that the rate constants were never "constant" and a different hydrolytic kinetics should have been there to model the data.

A diverse array of empirical and a few mechanistic model are available in literature for substrate utilization kinetics which are either coupled or uncoupled with microbial growth [17], [18]. Most of the proposed empirical growth models had evolved in modification to original Monod model either incorporating additional constants/parameters to account for maintenance, or the dependence of specific growth rate on the biomass density [19], [20]. In sigmoidal growth kinetics, empirical models such logistic model, Gompretz equations [21] and their modifications [22]–[24] or combinations of logistic model and Michaelis–Menten [25] models have been adopted. On other hand based on initial substrate/biomass ratio, the depletion profiles could be either logistic, first order, Monod (growth) or Monod (non-growth) [26].

The objective in present study was to evaluate the interdependency of microbial growth and ureolysis kinetic from set of designed batch experiments. The experimental data for growth and ureolysis were screened through a sets of independent growth and substrate depletion kinetics where all experimental profiles (for growth/ureolysis) were simultaneously regressed with modelled one. The best fit model for either growth or ureolysis when available, it was

concatenated with proper stoichiometric or kinetic relationship for complementary rate expression and was subjected to regression with entire data set. This data driven model selection and fitting approach could be valuable in reframing ureolysis driven biogeochemical modelling.

II. Materials and methods

A. *Microorganism and culture condition*

B. pasteurii (ATCC, 6453) used in this study were routinely maintained and grown on NH_4-YE medium containing (per litre) yeast extract, 20.0 g; $(NH_4)_2SO_4$, 10.0 g; 0.13 M Tris buffer (pH 9.0). All media components were autoclaved separately (for 15 min at 15 psi and 121°C) and mixed before inoculation. Culture was grown aerobically in thermostatic shaker-incubator set at 30°C, 150 rpm.

B. *Preparation of ureolysis broth*

Experiment was conducted in three different sets of 280 ml serum bottle (Wheaton) with 100 ml sterile (autoclaved) NB-NaCl media containing beef extract (3 g/L), peptone (5 g/L), and NaCl (5 g/L) pre-adjusted to pH 6.5. Urea from filter-sterilized (0.22 µm) stock was added into the serum bottle at their respective final concentrations as per design (Table 1). Overnight grown *B. pasteurii* culture was centrifuged at 3500*g* (10 min), supernatant was decanted and cell pellet was washed twice with sterile NaCl (5 g/L) and after a final spin, the pellets were transferred into respective bottles. Bottles were then capped with silicon stopper and incubated in an incubator-shaker (30°C and 160 rpm). Samples were withdrawn for measurement of free cell concentration, urea concentration.

TABLE I. EXPERIMENTAL DESIGN ADOPTED FOR UREOLYSIS KINETIC ANALYSIS

Sl. No.	Initial urea conc. (M)	Initial biomass concentration (g/l)
Set-1	0.1	0.25
Set-1	0.2	0.25
Set-3	0.1	0.15

C. *Biological and chemical analysis*

1) Biomass analysis

Optical density of the carefully drawn samples was measured at 600 nm using a UV-visible spectrophotometer (S-3100, Scinco Co. Ltd, Korea). The measurements were converted into dry weight basis biomass concentration (in g/l) using a standard calibration prepared in the identical range of concentrations.

2) Urea analysis

Urea concentration in the samples were determined by the modified thiosemicarbazide method [27]. Briefly, to 0.1 ml centrifuged sample supernatant, 0.8 ml of distilled water and 1 ml of 10% TCA were added. The mixture preparation were agitated for 2-5min and centrifuged, thereafter 0.1 ml of supernatant was transferred into test tube. To each of the tubes 5 ml of colour reagent (mixture of diacetyl monoxime, thiosemicarbazide, and ferric chloride in sulphuric acid-phosphoric acid solution) was added, mixed and immersed in boiling water bath for 10 minutes. After cooling down, sample absorbance at 520 nm was measured in UV-vis. spectrophotometer. Urea concentrations in the samples were calculated from calibration prepared with known standard, processed in identical way.

III. Kinetic model selection

A. *Choice of kinetic model for Biomass growth and ureolysis*

The experimental data *viz.* biomass and urea concentration profiles were utilized in the modelling process. Different kinetic formulations of varying complexity and underlying mechanism have initially been adopted in this exercise and the representative model equations have been presented in Table 2.

Primarily all the chosen models were of two different categories, *viz.*, first variant where rate expression for growth or substrate utilization are not coupled (either $dX/dt=f(X)$ or $dS/dt=g(S)$) henceforth termed as "independent model" and the other variant where rate expressions are intrinsically "coupled" ($dX/dt=f(X,S)$ or $dS/dt=g(S,X)$). In contrast to Monod based equations [28], the independent growth models offered a possibility to express growth rate independent of substrate concentration. When the rate expression for either growth or ureolysis is explicitly described in model (in Table 2), the complementary expression was framed utilizing the correlation as given in Eq. (1), with yield coefficient (m) and maintenance requirement ($Y_{X/S}$) of growing culture [20].

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - mX \quad (1)$$

In independent models for biomass growth exponential, logistic, modified logistic, Gompretz models and for ureolysis 1st order or pseudo-1st order kinetics were chosen. A logistic growth model could be plausible where growth slows down with increasing population size as available resources get limited [29].

On other hand the three-parameter modified Gompretz model function [28], [30], based on an exponential relationship between specific growth rate and population density, has widely been used to describe cell growth in a simple, yet adequate way [31]. Though inclusion of explicit "lag phase" parameter in modified Gompretz model makes it attractive for modelling an individual growth curve, but this parameters is not universally constant and can vary with substrate to biomass ratio. For ureolysis, first order kinetics remained most convenient choice in literature [11], [13], followed by pseudo first order (function of substrate and initial biomass concentration).

Out of the different coupled models, the Monod model explicitly utilizes the concept of a single growth limiting substrate [32]. Contois kinetics of growth [19] and substrate utilization [33] is of choice if hydrolysis rate could have been influenced by substrate to biomass ratio.

TABLE II. THE DIFFERENT FUNDAMENTAL KINETIC EXPRESSIONS ADOPTED IN THIS MODELING STUDY

Sl. No.	Model type	Name	Kinetic expression*
For biomass growth			
1	Individual	Exponential	$dX/dt = \mu X$
2	Individual	Logistic	$dX/dt = \mu X\left(1-\left(X/X_{\infty}\right)\right)$
3	Individual	Modified logistic	$dX/dt = \mu X\left(1-\left(X/X_{\infty}\right)\right)\left(1-\left(X_{\min}/X\right)\right)^{c}$
4	Individual	Gompretz	$dX/dt = \mu_0 X e^{-kt}$
For ureolysis			
5	Individual	1st order	$dS/dt = -k_1 S$
6	Individual	Pseudo first order	$dS/dt = -k_1^{'} S X_0$
For growth & ureolysis			
7	Coupled	Contois equation	$dX/dt = \mu'_{\max}\left(S/\left(k_x X + S\right)\right)X$
8	Coupled	Monod (growth)	$dX/dt = \mu'_{\max}\left(S/\left(k_s + S\right)\right)X$
9	Coupled	Second order	$dS/dt = -k_2 S X$

* X - Biomass concentration, X_0 – initial biomass conc., X_∞ - maximum carrying capacity, X_{min} - minimum biomass concentration, μ - specific growth rate, μ_0 is initial specific growth rate at time t=0, μ'_{max} is observable maximum specific growth rate, S - substrate concentration, concentration, k_1 and $k_1{}'$ are first order and pseudo-first order ureolysis rate constants, *Ks* - half saturation constant for Monod model, k_2 - second order rate constant

B. *Numerical approach adopted in modeling*

Equations for dS/dt and dX/dt for a given model were integrated using Matlab® with Runge Kutta (RK4) ODE solver. The biomass and ureolysis data from all the three experimental sets were regressed (allowing the model kinetic parameters to vary) with different independent models to maximize combined sum of regression coefficient (R^2_{Tot}) for entire set of growth (biomass) and/or ureolysis profile as given in Eq. 2.

$$R^2_{Tot} = \sum_{i=1}^{3}\left(1-\frac{\sum_{j=1}^{n}\left(X_{i,j}^{\exp}-X_{i,j}^{\mathrm{mod}}\right)^2}{\sum_{j=1}^{n}\left(X_{i,j}^{\exp}-\bar{X}_i^{\exp}\right)^2}\right) \quad (2)$$

Where, $X_{i,j}^{\exp}$ and $X_{i,j}^{\mathrm{mod}}$ are experimental and modelled data respectively for i-*th* set and j-*th* data point and $\bar{X}_i^{\exp}$ is experimental data average for i-*th* set. However, when both ureolysis and biomass growth models were simultaneously regressed, sum of all six regression coefficients (3 growth and 3 biomass profiles) were optimized.

IV. Results and discussion

A. *Ureolysis and biomass growth - batch experiments*

The ureolysis and biomass growth profiles have been presented in Fig.1. It is evident from the figure that there was an appreciable lag phase before active ureolysis initiate in different batches. About 62%, 42% and 52% of original urea were hydrolysed in set-1, 2, and set-3 respectively. Interestingly, even with same initial urea concentration (as in set 1, 3), the ureolysis profiles were markedly different. However, when starting with same initial inoculum concentration, the biomass growth profiles were identical even if they received different urea dosing. This also suggests that

in tested range of urea and biomass concentration, though ureolysis kinetic is somehow dependent on biomass concentration but not vice-versa. Some earlier studies have reported urease from *B. pasteurii* to be of extracellular in nature, but still remains contradictory as others have identified the enzyme to be cell-associated [34].

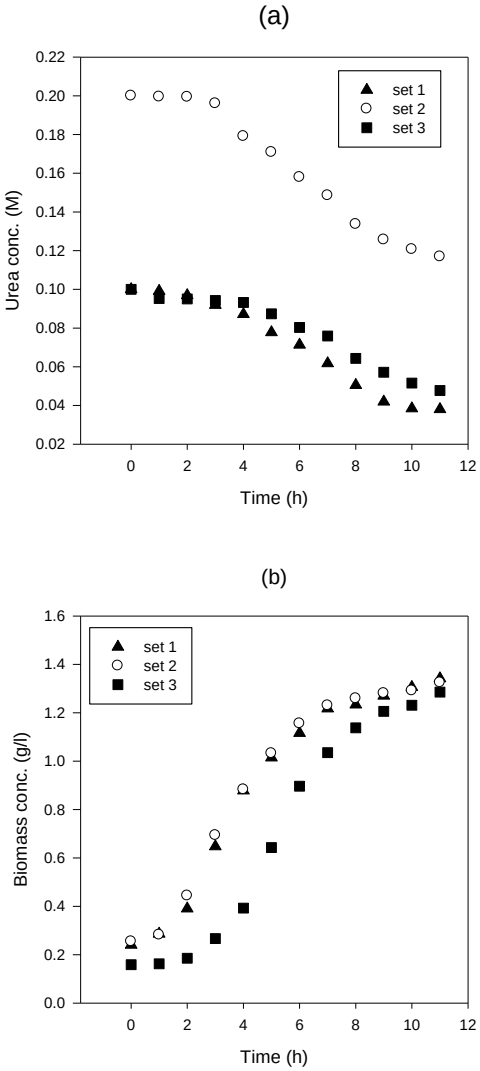

Figure 1. Ureolysis and biomass growth profile in different batch experimental system.

B. *Correlation between Ureolysis and growth*

The relation between biomass growth and ureolysis has been shown in Fig. 2. It is apparent from the figure that the slope ($\delta X/\delta S$) continued to decrease over time, where ureolysis continued without any proportional increase biomass concentration and the two cannot simply be correlated with a constant unique yield coefficient ($Y_{X/S}$). This phenomenon could possibly be attributed to any extracellular urease that would have leaked from microbial cell [35], or the continuous increased cellular maintenance requirement with increasing cell density. The maintenance requirements of microbial biomass represent the additional consumption of substrate, and some studies presume that growth is a secondary feature of energy utilization [36]. Similar observations have been reported in many earlier studies relating microbial growth on soluble substrate [37], [38].

C. *Modelling ureolysis and biomass growth*

1) Assessment of primary independent models

The modelling of experimental growth and ureolysis data to the individual kinetic models has been shown in Table 3. It is evident from the analysis that the logistic model and better modified logistic models substantiated the microbial growth (R^2>0.9 for each profile). However, the experimental and modelled profiles have much higher deviations arising out of the initial lag phase particularly in batch 3 with low initial biomass concentration (data not shown). As all three growth profiles are combinedly regressed with a single set of kinetic parameters, the deviations out of single profile becomes more prominent. It should be noted as logistic equation generates as a convex curve consisting of a monotonously increasing portion and a final stabilizing upper limit to population density [39] but lag phase at the initial period remains unaccounted [23].

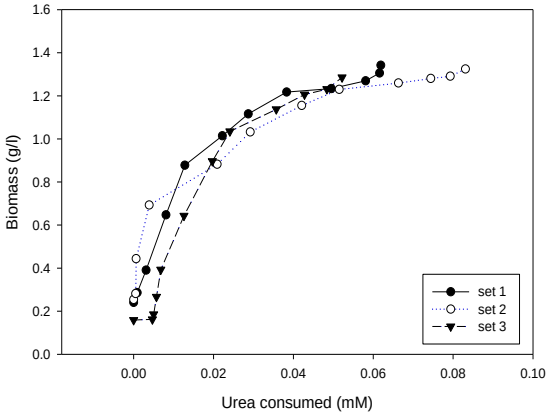

Figure 2. Correlation between urea consumed and biomass growth across experimental sets

When experimental growth data were modelled in modified logistic equation, as proposed by [24], overall regression coefficient got significant improvement which is attributed to the additional shape-function that

dampens the growth rate particularly at the observable lag phase.

In view of ureolysis kinetics, the two independent primary models (1st order and pseudo first order) largely failed to fit the experimental data. However, this is in line of our experimental observation, where ureolysis rate has been shown to vary with initial biomass concentration as well as growth rate (Fig. 1.). In literature, ureolysis rate has often been assumed to be first order in field and laboratory scale studies [14], [16]. The logic over simplified approach could possibly be valid provided at very high biomass/urea concentration ratio.

TABLE III. ESTIMATED PARAMETER AND REGRESSION COEFFICIENT FOR DIFFERENT PRIMARY KINETIC MODELS

Modeling approach	Model expression [c]		Estimated model parameters	Regression coefficient (R^2)		
	Growth (dX/dt)	*Ureolysis (dS/dt)*		*Set-1*	*Set-2*	*Set-3*
Individual	Exponential	-	$\mu = 0.18$	0.51	0.44	0.61
	Logistic	-	$\mu = 0.45$, $X_\infty = 1.41$,	**0.98**	**0.98**	**0.94**
	Mod. Logistic	-	$\mu = 0.89$, $X_\infty = 1.29$, c=0.7	**0.99**	**0.99**	**0.99**
	Gompretz	-	$\mu_0 = 0.18$ $k = 0.19$	0.51	0.44	0.61
	-	1st order	$k = 0.0516\ h^{-1}$	0.74	0.84	0.84
	-	pseudo 1st order	$k = 0.232\ h^{-1}$	0.79	0.72	0.72
Coupled	Monod	-	$Y_{X/S}=17.11$, $\mu'_{max}=0.19$, $k_s = 0.005$, m = 0	0.92[a] 0.55[b]	0.91[a] 0.41[b]	0.97[a] 0.58[b]
	Contois	-	Yx/s=32.79, $\mu_{max}=0.31$, $k_x=0.12$, m= 0.004	0.96[a] 0.64[b]	0.95[a] 0.51[b]	0.97[a] 0.74[b]
	2nd order	-	Yx/s=32.79, $k_2 = 0.10$	0.67[a] -0.62[b]	0.27[a] 0.64[b]	0.62[a] -0.34[b]

a – for urea profile, b – for biomass profile, c – When one of differential expression for growth or ureolysis is given by formal kinetic model and the complementary one is formulated by stoichiometric correlation: $dS/dt = -(1/Y_{X/S})dX/dt - mX$

TABLE IV. ESTIMATED PARAMETER AND REGRESSION COEFFICIENT FOR SELECTED GROWTH MODELS WHEN COUPLED WITH UREOLYSIS MODEL

Model For		Estimated model parameters	Regression coefficient (R^2)		
Growth	*Ureolysis*		*Set-1*	*Set-2*	*Set-3*
Mod. logistic	$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - mX$	$Y_{X/S}=143.8$, $\mu=0.89$, $X_\infty=1.29$, m =0.006, c =0.69	0.99[a] 0.97[b]	0.99[a] 0.91[b]	0.99[a] 0.98[b]
Mod. logistic	$dS/dt = k_2' X\left(\frac{S}{k_s + S}\right)$	$\mu = 0.89$, $X_\infty=1.29$, c=0.69, $k'_2=0.011$	**0.99[a]** **0.99[b]**	**0.99[a]** **0.98[b]**	**0.99[a]** **0.98[b]**

[a] – for urea profile, [b] – for biomass profile, *c* – Maximum substrate depletion rate (v_{max}) in original Michaelis-Menten expression has been substituted into as a function of biomass concentration. As $v_{max} = k_2 E_T$ and total enzyme content $E_T \propto X$; thus $V_{max} \propto X$

2) Assessment of the coupled models

The four different coupled models having explicit expression for growth rate (Contois, Monod growth) or substrate utilization rates (second order) were evaluated and results have been presented in Table 3. It is evident from results that both Monod and Contois growth models were not appropriate to represent biomass growth profiles. Monod growth equation is normally suitable for describing substrate limited growth at low cell populations [40] and unless at a very high substrate concentration, the assumption of Monod equations may not be valid in substrate hydrolysis and biomass growth [41]. On other hand, Contois model is most suitable for high cell density culture where specific growth rate proportionally decreases with biomass concentration. So, the Contois model cannot effectively illustrate the lag phase in growth profiles.

3) Modified logistic growth and conjugated substrate utilization

The modified logistic model has already been shown to be the best representative individual growth model in microbial ureolysis (all R^2>0.99). Two different rate expressions for substrate utilization were chosen with the modified logistic model and the overall regression results have been shown in Table 4. The experimental and modelled profiles are shown in Fig. 3. It is obvious from the figure that the modified logistic expression adequately simulate the biomass growth profiles in entire data set. It is evident from the result that when maintenance based correlation [20] was chosen, the predicted ureolysis profiles deviated from the experimental one with optimal regression coefficients of 0.97, 0.91 and 0.98 for experimental set 1, 2 and 3 respectively. Constrain for unique biomass yield coefficient in optimization procedure cannot simply account the differential amount of substrate

utilization with an unique asymptotic biomass concentration across different batches. However, when Michaelis–Menten (M-M) kinetics conceptualizing enzymatic hydrolysis was considered, the experimental and modelled ureolysis were in well agreement with each other. As Michaelis–Menten kinetics formulation supports substrate depletion without unlinked to active biomass growth [42], it can explain continued ureolysis even when biomass concentration approaches its asymptotic value, as in present study. M–M models though common for the description of pure enzyme kinetics (including urease) but have been widely applied to systems with ureolytic bacteria, as well [34], [43], [44]. However, original interpretation of maximum substrate depletion rate as in M-M model is no longer valid here, as total enzyme content (which is assumed to be proportional to biomass) increases with time and is not a constant. In this study the growth model is assumed to be independent of the ureolysis models but the ureolysis models are scaled by the population density. A similar approach has also been adopted in a recent study [44], where authors first fitted the population growth with a Gompretz model independently of the ureolysis one. Thus adoption of two discrete models for microbial growth and ureolysis could be valuable choice rather than un-necessary over-simplification approach.

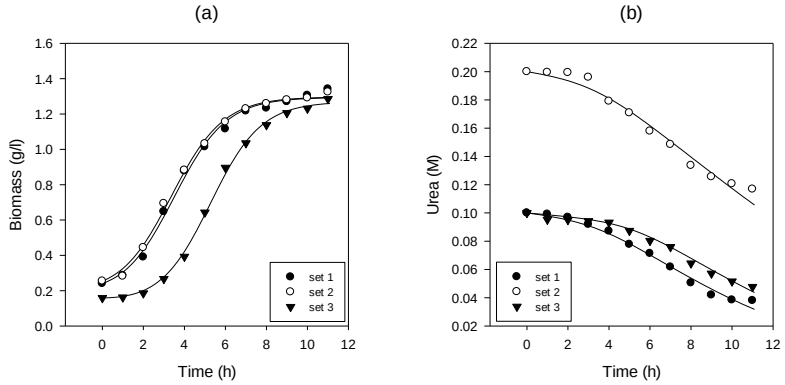

Figure 3. Comparison of experimental and modified logistic model predicted growth and ureolysis profile in different batch experiment.

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