

Kinetic Measurements for *Pseudomonas aeruginosa* MR01 During Biosurfactant Production in Two-phase System and Developing a Double-Exponential Model for Viable Cell Profile

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Abstract: Biosurfactants are microbial substances which influence interfacial tension. The kinetic study was carried out for *Pseudomonas aeruginosa* MR01 during biosurfactant production in a two-phase liquid-liquid batch fermentation system. The maximum rhamnolipid concentration (P_{max}) and the yield of biosurfactant per biomass ($Y_{p/X}$) in a 5-L bioreactor containing soybean oil medium were found to be approximately 20.9 g.L⁻¹ and 3.1 g.g⁻¹, respectively. Previously reported kinetic models in aqueous systems, three-parameter Gompertz, Luedeking-Piret and Mercier equations, demonstrated adequate goodness-of-fit ($r^2 > 0.9$) to kinetic data recorded for biomass growth, nitrate consumption and rhamnolipid formation during fermentation. A double-exponential equation was developed to model time-course data of viable cells reported as colony-forming units (CFUs). This suggested equation could accurately predict the experimental data with $r^2 = 0.947$ and provide a powerful expression to plot microbial cell count pattern. Low values of the standard error of estimate (SEE) and chi-square (χ^2) confirmed the acceptable level for the accuracy of fit. The 95% confidence intervals for kinetic parameters in all nonlinear models indicated a narrow range simulating the precision of estimation. This kinetic study may work efficiently in the design and scaling-up of the coming aqueous-organic batch bioreactors which are to be applied to the production of biosurfactants.

Key words: Batch fermentation • Kinetic behavior • Modeling • Biosurfactants • two-phase system

INTRODUCTION

Biosurfactants are valuable microbial compounds with a wide range of surface-active features. They may be used as foaming, wetting, solubilizing, antiadhesive and antimicrobial agents, emulsifiers, immune regulators and immune modulators. They are potential candidates for many commercial applications in the petroleum, pharmaceutical, biomedical and food processing [1-3]. Among principal surfactant groups, rhamnolipids produced by *Pseudomonas aeruginosa* strains have been the most widely studied. Previous studies on rhamnolipid production by *P. aeruginosa* under different conditions that enhanced production yield, have indicated that the quality and quantity of the produced rhamnolipids are

influenced by several factors, such as nitrogen and ion concentration, pH, temperature, aeration rate and the nature of the carbon source [4]. However, a lack of comprehensive perception of the rhamnolipid production kinetics and behavior of *P. aeruginosa* cells under controlled conditions at the bioreactor scale and the absence of respective comparative studies are among the key issues regarding insufficient productivities and cost-effective production of these biosurfactants [5]. Several studies have shown that the higher yields of rhamnolipid are obtained when *P. aeruginosa* is cultivated on water immiscible substrates as carbon sources, when compared to the miscible ones [6-8]. Nevertheless, kinetic models have only been presented for rhamnolipid production with water soluble carbon

sources, but most recently Medina-Moreno *et al.* suggested a model to describe the microbial production of biosurfactant using oleic acid as a substrate immiscible in water [4]. However, both Monod-type and sigmoidal models such as logistic and Gompertz have previously been applied to biomass kinetics [9-12]. Williams and Luedeking-Piret developed substrate consumption equations which have been widely used in bacterial fermentation [13-14]. Several equations have been previously established to model microbial production [15-17]. The present work proposed to monitor rhamnolipid production by *P. aeruginosa* MR01 using soybean oil in a controlled bioreactor system. Moreover, it focused on the kinetic modeling of microbial production and growth at the bioreactor scale. In this study, 3-parameter Gompertz and Leudeking-Piret equations used by Chavez-Parga *et al.* [14] were applied to model the time-course pattern of biomass growth and substrate utilization, respectively. Additionally, time-course profile of rhamnolipid production was modeled by the equation of Mercier *et al.* [15]. Furthermore, a novel kinetic model which was not addressed by previous works was derived for modeling the viable cell pattern over time, which was based on a double-exponential equation. Developing a model for the time-course profile of viable *P. aeruginosa* cells is a matter of great importance in demonstrating upward trend of rhamnolipid while biomass growth ceases and viable cell counts descend.

MATERIALS AND METHODS

Chemicals, Microorganism and Maintenance:

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soybean oil was provided from the Shahrvand chain stores Inc. (Iran). The *Pseudomonas aeruginosa* MR01 (EU795302) strain, originally isolated from crude oil in a previous work [18], was used throughout this study. The bacterial cells were maintained as glycerol cultures (70:30 (v/v) mixture of freshly grown cells and 0.85% (v/v) glycerol solution in water) at -70°C.

Inoculum Preparation: To prepare an inoculum, a 1 mL sample from the glycerol stock culture of *P. aeruginosa* MR01 was inoculated into 7 mL of lysogeny broth, LB, [19] in a 50 mL-falcon tube and incubated overnight at 30°C, with shaking at 200 rpm. Thereafter, a total volume of 1 mL was transferred to 50 mL of LB broth in a 250 mL-shake flask and kept overnight at 30°C and 200 rpm. Cell pellets were first collected by centrifugation of the

culture medium at 2,147×g for 20 min and then suspended in a specified volume of soybean oil medium (SOM) [8] to reach an optical density of (OD_{600}) of 1. Appropriate volumes of the seed cultures served as inoculum for starting up the batch cultivation in the bioreactor.

Culture Medium Composition and Cultivating

Conditions: All cultivations were carried out at 30°C in a 5-L stirred tank bioreactor (New Brunswick Bio Flo III, USA) containing 3.5 (l) of SOM and 2.5% (v/v) inoculum. The bioreactor was equipped with an integrated process control system for temperature, pH, pO₂ and airflow. Foaming was controlled manually by raising and lowering the aeration and agitation rates inversely. To control the foam level and avoid overflowing the bioreactor, aeration rate was reduced from 1 to 0.5 volume of air per volume of liquid per min (vvm) and so agitation rate was raised from 250 to 300 rpm, to compensate for drop in the dissolved oxygen (DO) and keep it higher than 10%. Indeed, an extra impeller with rectangular blades angled at 45°, was mounted on a common axis with the blending impeller positioned at a distance of about 8 cm from, so as to break the foam that was produced. Moreover, one drop of antifoam was added when foam volume filled all the void spaces above the liquid level in the bioreactor.

Sampling and Analytical Methods:

At certain time intervals, samples were withdrawn for offline analysis of bacterial growth, biosurfactant production and nitrate consumption. Viable cell counts (CFU per milliliter) were estimated by plating the appropriate bacterial serial dilutions onto LB agar and incubating the plates at 37°C for 24 h. The culture suspension was mixed vigorously with n-hexane 1:1 (v/v) and centrifuged (4,830×g, 4°C, 30 min) to separate the biomass, aqueous and organic solvent [5]. The organic solvent phase was removed and the cell pellets were washed once with physiologic serum solution, centrifuged (4,618×g, 4°C, 30 min) and dried till constant weight. Nitrate concentrations were determined in the aqueous phase by applying the slightly modified method of Jagessar and Sooknundun [20], in which ammonium hydroxide solution was replaced with 12N potassium hydroxide solution [21]. To avoid interference by nitrite during nitrate analysis, nitrite concentration levels should be kept below 0.2 mg.L⁻¹. Hence, nitrate concentrations were measured in suitable serial dilutions of the aqueous phase whose nitrite concentration was checked by the Ivanov spectrophotometric method using a mixture of sulfanilic acid and 1-naphthylamine [22]. Rhamnolipids were quantified in the aqueous phase using

an acid precipitation and solvent extraction method that employed ethyl acetate [23]. All assays were conducted in triplicate. Accordingly, the data presented here are the arithmetic averages of at least three replicates and the error bars represent the standard deviations.

Data Processing and Mathematical Model Description:

Experimental data were fitted to the proposed models to estimate the kinetic model parameters using MATLAB 7.1 by minimizing nonlinear least squares curve fitting. A numerical integration command in MATLAB, known as ode45, was applied to solve the differential equations using the Runge-Kutta technique. Although the logistic model has been widely used for cell mass growth kinetics, it did not work well in this study. A modification of the classical function that was introduced by Gompertz for population growth, in 1825 [24] and the 3-parameter Gompertz equation used by Chavez-Parga *et al.* [14], were applied to mathematically model the biomass growth as the following expression (Eq.1);

$$\frac{dX}{dt} = kX^{-\mu} - aX \quad (1)$$

where X is the biomass concentration (g.L^{-1}), k and a are kinetic parameters relating to initial specific growth rate (h^{-1}) and growth inhibition (h^{-1}), respectively. μ implies the specific growth rate (h^{-1}) and t denotes the time (h). Nitrate consumption by *P. aeruginosa* MR01 was interpreted by the Luedeking-Piret differential equation that neglects the amount of substrate used for product formation [14]. The equation is given below as Eq.2:

$$\frac{dN}{dt} = -\frac{1}{R_{X/N}} \frac{dX}{dt} - m_N X \quad (2)$$

where N is nitrate concentration (g.L^{-1}), $R_{X/N}$ refers to ratio of biomass to nitrogen and m_N represents the maintenance coefficient (h^{-1}) indicating the amount of nitrate needed for energy, or maintenance requirements.

The kinetics of rhamnolipid formation was based on the equation suggested for lactic acid production [16] and is presented below as Eq. 3:

$$\frac{dP}{dt} = P_r P (1 - P / P_{\max}) \quad (3)$$

where P is the biosurfactant concentration (g.L^{-1}), P_{\max} shows maximum concentration of biosurfactant (g.L^{-1}) and P_r is the ratio of the initial volumetric production rate (r_p) to the initial product concentration P_0 (h^{-1}).

Developing a Double-exponential Model for Viable Cell Kinetics:

Although both viable cells and biomass are of the same type, the trend in viable cell counts was partly unlike the biomass profile. The trend in cell mass (Fig. 1) appeared to have increased exponentially, similar to that described by the Gompertz equation. The viable cell pattern presented in Figure1 can be divided into two stages. The first stage, expressing cell growth showed a constantly increasing curve and the second one indicating cell lysis, represented a constantly decline without any apparent S-shape. As a result, a single exponential function of time could not fulfill

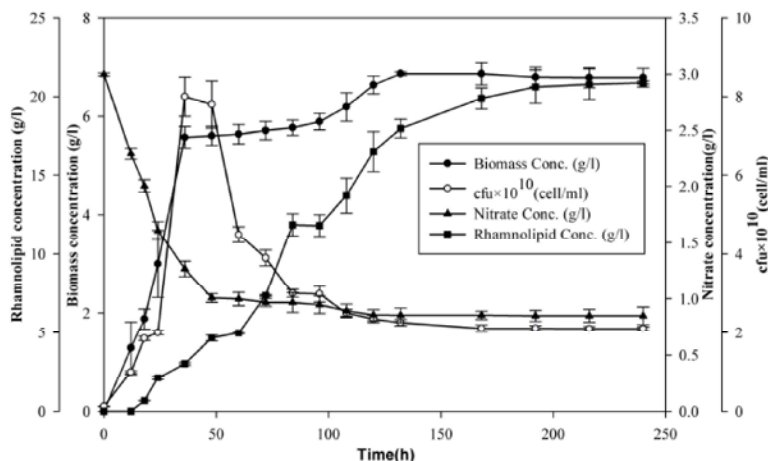


Fig. 1: Time-course profile of biomass growth, viable cell counts, nitrate consumption and rhamnolipid production during *Pseudomonas aeruginosa* MR01 growing in SOM. Results represent the average of three independent experiments.

the time-course profile of viable cell counts showing a non-monotonic pattern over time. However, mathematical modeling of viable cell kinetics is a challenging problem in mathematical biology and encourages developing a new mathematical method. In order to overcome this problem, viable cell kinetic data were fitted to a double-exponential expression of the form shown in Eq. (4), by a least squares technique, using MATLAB:

$$\frac{dc}{dt} = k_g c e^{-\sigma_g t} + k_l c e^{-\sigma_l t} - a_c c \quad (4)$$

where c is the viable cell count (cell/mL), k_g and k_l are kinetic parameters pertaining to initial specific growth in cell number and lysis rates (h^{-1}), respectively. σ_g and σ_l are the specific growth in cell number and lysis rates (h^{-1}), respectively and a_c is a kinetic parameter relating to cell count inhibition (h^{-1}).

Statistical Analysis: Quantitative evaluations were carried out to verify the validity of the proposed models. Standard error of estimate (SEE) and chi-square (χ^2) were estimated to check on the consistency between the experimental kinetic data and corresponding model-predicted values [25]. Confidence intervals were considered to examine the reliability of the estimated parameters. The MATLAB built-in functions were used to perform statistical analysis.

The standard error of estimate, as a measure of error during prediction, was employed to indicate how accurately each equation predicts relevant values. This is illustrated in Eq. 5 [25-26]:

$$SEE = \sqrt{\frac{\sum_{i=1}^n (M_{i,exp} - M_{i,pre})^2}{d_f}} \quad (5)$$

where $M_{i,exp}$ and $M_{i,pre}$ are experimental and model predicted values at the i^{th} data, respectively. n is the number of data points and d_f is the degree of freedom or the number of independent variables in the regression model.

The chi-square (χ^2) test was applied to determine the goodness of fit between the observed and calculated data. Chi-square is the sum of the squared difference between experimental (exp) and predicted data (pre) divided by the predicted data in all possible categories [25]. The mathematical equation is given as Eq. 6 [25, 27]:

$$\chi^2 = \sum_{i=1}^n \frac{(M_{i,exp} - M_{i,pre})^2}{M_{i,pre}} \quad (6)$$

To meet the model robustness, χ^2 should not exceed the corresponding critical value shown by χ_c^2 , as presented in Eq.7 [25]:

$$\chi_c^2 = \chi_{1-\alpha}^2 (n-1-\theta) \quad (7)$$

where n is number of data points, θ and α are the number of model parameters and significance level, respectively. Assuming that the level of significance (α) is 0.05 and with respect to the number of data points in this study (17), χ_c^2 is calculated by $\chi_{0.95}^2 (16-\theta)$ regarding the number

of parameters for each model. The confidence intervals of the best-fit values of nonlinear regression were provided through the MATLAB statistics toolbox. The confidence intervals reflect the uncertainty associated with the parameter estimate or in other words, the precision of a parameter estimate. A 95% confidence interval means that the true value of the fitting parameter has a 95% probability of falling within the confidence interval [24].

RESULTS AND DISCUSSION

Kinetic Behavior in the Batch System: Rhamnolipid production by *P. aeruginosa* MR01 was accomplished three times, in a 5- L bioreactor system using soybean oil as the sole carbon source. The typical time course profile of nitrate consumption, cell growth and rhamnolipid production are depicted in Figure 1. Based on these results, most of the nitrate was consumed during the first 48 h of cultivation when bacteria grew exponentially and generated a biomass of 5.6 g L^{-1} (Fig. 1). Thereafter, cells entered stationary phase but unexpectedly cell mass revealed a growing pattern with a gentle slope after 96 h and followed a flat and constant trend after 132 h. It was coupled with a mild decrease in the number of viable cells and steep increase in rhamnolipid production between 96 h and 132 h, during the cultivation period. This phenomenon seems to have originated from cell lysis and release of biosurfactant and other components into the culture medium. Substantial rhamnolipid production was detected at the end of the exponential phase of growth, as also demonstrated by Nitschke *et al.* [28]. This might be traced back to the absence of nitrate ions, reaching a specified carbon-to-nitrogen ratio [29] and/or attaining the high cell density which is a requisite factor for rhamnolipid expression by *P. aeruginosa* through the quorum sensing mechanism [30-31]. Rhamnolipid

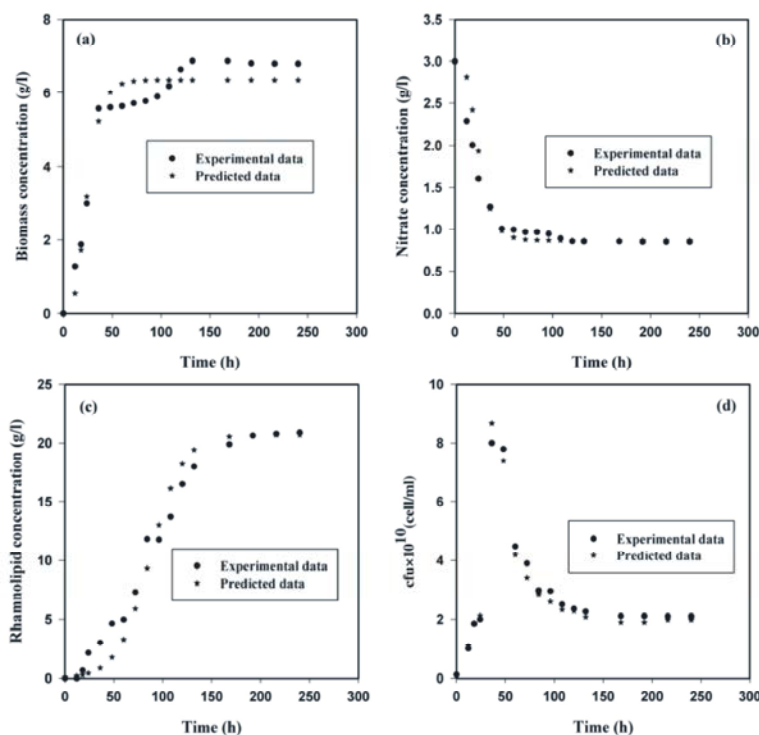


Fig. 2: Representation of experimental and predicted data for (a) biomass growth, (b) nitrate consumption, (c) rhamnolipid production and (d) viable cell counts during *Pseudomonas aeruginosa* MR01 growing in SOM. Predicted data showing in figures (a-d) were calculated by using the equations (1-4), respectively. Results represent the average of three independent experiments.

Table 1: Model parameters estimated by minimizing nonlinear least squares curve fitting of kinetic data for *Pseudomonas aeruginosa* MR01 growing in SOM^a

Biomass growth				Nitrate consumption			Rhamnolipid production			Viable cell counts					
k (h ⁻¹)	μ (h ⁻¹)	α (h ⁻¹)	r^2	$R_{X/N}$ (g/g)	m_N (h ⁻¹)	r^2	P_r (h ⁻¹)	P_{max} (g l ⁻¹)	r^2	k_g (h ⁻¹)	σ_g (h ⁻¹)	k_i (h ⁻¹)	σ_i (h ⁻¹)	a_c (h ⁻¹)	r^2
0.926	0.106	0.001	0.958	2.986	0.001	0.934	0.081	20.8	0.933	22.61	0.026	-22.37	0.024	-0.0096	0.947

^a Parameters defined in text.

production continued up to 168 h of cultivation and subsequently ascended subtly to a maximum of approximately 20.9 g.L⁻¹, after 200 h. Visually, after 24 h of cultivation, foam started to appear, which seemed uncongested at first, but increasingly evolved into the compact form with the progress in cultivation, leading to a flooding situation in the bioreactor system despite the action of the mechanical foam breaker. To curb the excessive foam and prevent the bioreactor from overflowing, a drop of antifoam was added to the system at approximately 26, 90 and 115 h of cultivation, when foam rose in the fermentation vessel.

Determination of Kinetic Parameters: Figure 2 (a-d) presents the observed data as well as the predicted values calculated by Eqs. (1) - (4) for *P. aeruginosa* MR01 growing in SOM medium.

Experimental data were fitted to relevant models using the nonlinear least squares regression. The regression parameters were outlined in Table 1, which show that the mathematical models describe the experimental data realistically, with r^2 of 0.958 and 0.938 for biomass growth and nitrate consumption, respectively. The $R_{X/N}$ value of 2.986 g.g⁻¹, as estimated by Eq.2 was comparable to the experimentally calculated value of $R_{X/N} = \frac{\Delta X}{\Delta N}$ (3.16 g.g⁻¹),

where ΔX and ΔN are differences between the initial and final biomass and nitrate concentrations, respectively. A low maintenance coefficient (m_N) of 0.001 h⁻¹ indicated that the energy of substrate metabolism which is not involved in biomass synthesis was small, thus leading to a high cell density during nitrate depletion. Figure 2c affirmed that the Mercier model described the time course profile of rhamnolipid production well, with an r^2 of 0.933,

Table 2: Validating measures estimated by statistical analysis of kinetic models parameters for *Pseudomonas aeruginosa* MR01 growing in SOM^a

Models	SEE	χ^2	$\chi^2_{0.95}(16-\theta)$	Parameters	95% Confidence interval	
					Lower bound	Upper bound
Biomass concentration	0.492	0.9056	22.4	k	0.84	1.0119
				μ	0.0954	0.116
				a	-0.0006	0.006
Nitrate consumption	0.199	0.3104	23.8	R_{XN}	2.4124	3.5602
				m_N	-0.0006	0.0006
Rhamnolipid production	2.04	6.084	23.8	P_r	0.0788	0.0943
				P_{max}	18.438	22.9621
Cell counts	0.3206	0.0601	19.7	k_g	17.681	25.423
				μ_g	0.0121	0.0329
				k_l	-35.41	-17.432
				μ_l	0.0123	0.0338
				a_c	-0.0001	0.0001

^a Parameters were identified in nomenclature α is the level of significance=0.05

but slight differences were also apparent at earlier times. Considering the results in Figure1, maximum rhamnolipid concentration (P_{max}) was 20.9 g.L⁻¹, which came quite close to 20.8 g.L⁻¹, as predicted by the Mercier equation. The yield factor in relation to biomass was experimentally obtained as approximately 3.1 g.g⁻¹ using the $Y_{P/X} = \frac{\Delta P}{\Delta X}$

equation and the results shown in Figure1, where ΔP and ΔX represent differences between the initial and final product and biomass concentrations, respectively. Fermentation by *P. aeruginosa* MR01 in SOM in a 5-L bioreactor showed a high biosurfactant yield when compared to $Y_{P/X}$ values reported in previous works [32-33]. The double-exponential model was found in all cases to provide a markedly excellent fit to the viable cell kinetic data (Fig. 2d), with $r^2=0.947$.

Kinetic Model Validation: Standard errors of estimate showing low values in Table 2 cleared the relative goodness of fit of the proposed models. The results of the chi-square (χ^2) analysis for different proposed models depicted in Table 2 were below the relevant critical chi-square (χ^2_c). Therefore, there was no significant difference between the experimental data and the ones generated by the models.

The narrow confidence intervals for the estimated model parameters presented in Table 2 connoted the low uncertainty of fitting parameter values. It was concluded that these values and model predictions are not very sensitive to the particular sample used for building the model. The width of the confidence interval is a measure of the uncertainty about the position of the true value of the estimated parameter and a wide confidence interval

means that data do not define that parameter very well. Consequently, the narrow widths at high confidence level are desirable. The narrowness of the obtained confidence intervals rendered the parameters to lie in the estimated confidence regions, with 95% contingency. Comparison between the results given in Tables 1 and 2 showed that the calculated values of the model parameters fell in the confidence zone.

CONCLUSION

The results (P_{max} , $Y_{P/X}$) obtained for *P. aeruginosa* MR01 growing in SOM (in a 5-L bioreactor) under controlled conditions, demonstrated this strain as a strong biosurfactant producer as compared to the previously reported rhamnolipid producers. The proposed models were capable of accurately predicting the experimental results pertaining to cell growth, nitrate utilization and rhamnolipid production, with $r^2>0.9$. Time course profiles of viable cell counts were successfully modeled using the double-exponential approach, with $r^2=0.947$. The SEE and chi-square analyses revealed a rather excellent goodness of fit between the proposed models and the observed data. The width of the constructed confidence intervals were narrow and demonstrated that the true values of the fitting parameters would have a probability of 95% to lie in confidence region. In conclusion, all mathematical equations were statistically sufficient to describe the kinetic patterns for *P. aeruginosa* MR01 under the conditions mentioned above. Further studies should be carried out to establish the equations extending parameters to specify the mass transport effects and interface interactions.

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