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A Mathematical Modelling to Predict Rhamnolipid Production by *Pseudomonas aeruginosa* under Nitrogen Limiting Fed-Batch Fermentation

Seyed Ali Jafari, Mohammad Ghomi Avili, Emad Benhelal

Abstract**—**In this study, a mathematical model was proposed and the accuracy of this model was assessed to predict the growth of *Pseudomonas aeruginosa* and rhamnolipid production under nitrogen limiting (sodium nitrate) fed-batch fermentation. All of the parameters used in this model were achieved individually without using any data from the literature.

The overall growth kinetic of the strain was evaluated using a dual-parallel substrate Monod equation which was described by several batch experimental data. Fed-batch data under different glycerol (as the sole carbon source, C/N=10) concentrations and feed flow rates were used to describe the proposed fed-batch model and other parameters. In order to verify the accuracy of the proposed model several verification experiments were performed in a vast range of initial glycerol concentrations. While the results showed an acceptable prediction for rhamnolipid production (less than 10% error), in case of biomass prediction the errors were less than 23%. It was also found that the rhamnolipid production by *P. aeruginosa* was more sensitive at low glycerol concentrations.

Based on the findings of this work, it was concluded that the proposed model could effectively be employed for rhamnolipid production by this strain under fed-batch fermentation on up to 80 g l^{-1} glycerol.

Keywords**—**Fed-batch culture, glycerol, kinetic parameters, modelling, *Pseudomonas aeruginosa*, rhamnolipid.

I. Introduction

ALMOST all surfactants currently being used are chemically synthesized. However, the use of microbial surfactants (bio-surfactants) has steadily increased in recent years due to their diversity and environmental friendly nature [1]. Rhamnolipid is one of the most widely used bio-surfactant which is mostly produced during the stationary phase of growth of *P. aeruginosa* [2], [3]. Fed-batch cultivation is the most effective process strategy to achieve high productivity of rhamnolipid. It is due to the fact that stationary phase can be easily controlled by maintaining the concentration of substrates in the low levels in bioreactor [2]. Therefore developing a control strategy is a vital demand to provide the optimum conditions and to increase productivity [4].

Several factors such as substrate concentration, carbon to nitrogen ratio, feed flow rate, etc. can be optimized during fed-batch fermentation. However, doing trial and error experiments which considers a combination of all effective factors is very costly and time-consuming [5]. Therefore mathematical modelling of the process can be effectively used to compensate these deficiencies [6]. Moreover, a detailed kinetic study and proper estimation of the parameters is necessary to improve accuracy of the proposed model [7].

Employing mathematical modelling to optimize and to predict the biomaterial production is found in many studies. Guerra et al. designed the best feeding strategies using mathematical model to enhance fed-batch production of pediocin by *P. acidilactici* [8]. Model-based fed-batch operation was also very helpful to Khanna and Srivastava to over produce PHB by *R. eutropha* [9]. Guerra et al. developed a fed-batch mathematical model in order to design, scale-up, control, and optimize the production of probiotics [10]. Srivastava and Srivastava proposed a fed-batch model to predict suitable nutrient feeding strategy for better phosphate removal by *Acinetobacter calcoaceticus* [11]. Luna-Flores et al., Song et al., and Roosta et al. also presented several research works in this field [12]–[14].

The main objective of this study is to propose a mathematical model that relies on its own parameters to predict the amount of the rhamnolipid production by *Pseudomonas aeruginosa* under nitrogen limiting fed-batch fermentation. The estimated parameters are then introduced as reliable parameters for rhamnolipid production by this strain under fed-batch fermentation on glycerol. Eventually, several experiments are conducted to evaluate the accuracy of the proposed model.

II. Materials and Methods

A. Micro-Organism

Bacterial strain which was used to produce rhamnolipid bio-surfactant was *Pseudomonas aeruginosa* ATCC 53752. It was provided by Kerman University of Medical Sciences, Iran which was maintained on *Pseudomonas* agar (for pyocyanin, HIMEDIA) plate with composition of peptic digest of animal tissue 20, potassium sulphate 10, magnesium chloride 1.4 and agar 15 g l^{-1} in deionized water at 4°C. The pure colonies were sub-cultured every week on fresh *Pseudomonas* agar plate.

Seyed Ali Jafari is with the Department of Environment, Persian Gulf Research Institute, Persian Gulf University, Bushehr 75169, Iran (e-mail: sajafari.pgrsc@yahoo.com).

Mohammad Ghomi Avili is with the Department of Chemical Engineering, Isfahan University of Technology, Isfahan 84156-83111, Iran (e-mail: mgh98mgh@gmail.com)

Emad Benhelal is with the Department of Chemical and Materials Engineering, The University of Auckland, Symonds Street, 1010 Auckland, New Zealand, (e-mail: emad_benhelal_ce@yahoo.com).

B. Composition of Fermentation Medium

A basal mineral medium with composition of KH_2PO_4 3.4, K_2HPO_4 4.3, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.04 and $FeSO_4$ 0.04 g l^{-1} in deionized water was supplemented with trace element solution with the composition of $ZnSO_4$ 0.068, $CuSO_4$ 0.006, $NaMO_4$ 0.07, H_3BO_3 0.015 and $ZnCl_2$ 0.4g l^{-1} in deionized water. Each liter of the final fermentation medium contained 975 ml of the basal mineral medium and 25 ml of the trace element solution. Glycerol and sodium nitrate as carbon and nitrogen sources were added to the fermentation medium to achieve desired concentrations based on constant molar carbon to nitrogen ratio of 10 (C/N=10). This value for C/N was considered by taking the average of the best C/N values of 12 [15] and 8 [16] which had been previously reported as the optimum values for rhamnolipid production by *P. aeruginosa* with $NaNO_3$.

C. Fermentation Process

A bioreactor made of glass with one liter capacity filled with 500 ml fermentation medium was continuously aerated with the constant flow rate of 1 l min^{-1}. Air flow was also used to stir and homogenize the medium. Inlet air was sterilized using air filters and aseptic condition of fermentation broth was regularly verified by streaking a sample of the fermentation medium on a fresh *Pseudomonas* agar plate. A uniform and constant blue-green color of the plate during 24 hr of incubation at 37 °C implies an uncontaminated broth. pH of the fermentation broth was initially adjusted to 7.0±1 using 1.0 M HCl or NaOH prior to autoclaving. The inoculated mediums were incubated at 37±1°C by a water bath. The same overnight culture medium was used to inoculate the experiments (10% v/v).

Samples were withdrawn in specific time intervals followed by centrifugation (3461×g for 25 min) in order to analyze the precipitated biomass using dry weight method [15] as well as rhamnolipid determination in the supernatant using phenol-sulfuric acid method [15]–[18]. Rhamnolipid concentration was expressed as gram rhamnose per liter. A calibration curve was prepared with different concentrations of rhamnose of analytical grade (L(+)-Rhamnose monohydrate for biochemistry, Merck, Germany).

D. Theory

1. Kinetic Parameters Estimation

Several growth kinetic models were evaluated in order to estimate the best kinetic parameters of the *P.aeruginosa* growth (Unpublished data) which indicated that dual-substrate Monod model (1) had a better compatibility with the experimental data than the other models.

$$\mu = \frac{\mu_m . C_g . C_n}{(K_g + C_g).(K_n + C_n)} \quad (1)$$

where μ and μ_m are specific growth rate and maximum specific growth rate of microorganism (hr^{-1}), C_g and C_n are glycerol and sodium nitrate concentration (g l^{-1}) and K_g and K_n are half saturation constants for carbon and nitrogen sources (g l^{-1}), respectively. The unknown kinetic parameters in (1) were achieved by some assumptions as follows.

Thirteen batch experiments were conducted in triplicate at different glycerol concentrations (in the range of 0.1 to 30 g l^{-1}, C/N=10) with the same inoculum concentration of 0.22 g l^{-1} (dry weight) under predetermined pH and temperature. The specific growth rates were calculated individually in each runs based on (2), growth equation, as follows:

$$\frac{dx}{dt} = \mu x \quad (2)$$

where x is biomass concentration (g l^{-1}) and t is time (hr). The specific growth rate was considered approximately constant since the glycerol and sodium nitrate consumption was assumed negligible in the first few hours of the batch fermentation. Therefore, (3) obtained by integrating from (2) which is used to calculate the specific growth rate as a function of biomass concentration over time.

$$\mu = \frac{\left(ln \frac{x_t}{x_0}\right)}{\Delta t} \quad (3)$$

where x_0 and x_t are initial and final biomass concentration at time t in bioreactor respectively (g l^{-1}). The final biomass concentration was measured after 1.5 hours of fermentation.

The values obtained for specific growth rates at each pairs of glycerol and sodium nitrate concentrations were then fitted by dual-substrate Monod model (1) using LAB Fit software (V 7.2.48) to find the unknown kinetic parameters.

Other unknown parameters namely $Y_{x/g}$, $Y_{x/n}$ and k_d (biomass to glycerol and biomass to sodium nitrate yield (g g^{-1}) and endogenous respiration constant of microorganism (hr^{-1}), respectively) were estimated by solving the following equations simultaneously with finite difference numerical method using MATLAB programming software (R2009a 7.8.0). These equations ((4) to (8) [19]) were considered for biomass growth and glycerol and sodium nitrate uptake under batch fermentation process.

$$\frac{dx_a}{dt} = (\mu - k_d).x_a \quad (4)$$

$$\frac{dx_i}{dt} = 0.2\,.k_d.x_a \quad (5)$$

$$x_{Tot} = x_a + x_i \quad (6)$$

$$\frac{dC_g}{dt} = \frac{\mu . C_g}{Y_{x/g}} \quad (7)$$

$$\frac{dC_n}{dt} = \frac{\mu . C_n}{Y_{x/n}} \quad (8)$$

where x_a, x_i and x_{Tot} are active, inactive and total biomass concentration in bioreactor (g l^{-1}), respectively. As (5) shows, it was assumed that only 20% of dead cells could be considered as the measurable solid while the remaining were

usually decomposed to soluble materials which were consumed by active cells [20]. The trial and error method was applied to solve the above equations. Since biomass concentration is sensitive to initial guesses for $Y_{x/g}$, $Y_{x/n}$ and k_d, the search was constrained to a respective predetermined range of 0.01 to 3, 0.1 to 8 g g^{-1} and 0 to 2 hr^{-1}. The best trios of the parameters were judged according to the minimum value achieved for Absolute Average Relative Error (AARE) between experimental and theoretical x_{Tot}. It was calculated as given by (9):

$$AARE = \frac{1}{n}\sum_{i=1}^{n}\left(\left|\frac{X_{Tot,Exp,i} - Y_{Tot,Model,i}}{X_{Tot,Exp,i}}\right|\right) \quad (9)$$

where n is number of experimental samples at each run.

2. Fed-Batch Process Modelling

These set of experiments were evaluated by considering the effects of changes in initial glycerol concentration (20 and 60 g l^{-1}) as well as feed flow rate (10.42, 5.21 and 3.47 ml hr^{-1}). Feed was containing sterile sodium nitrate solution, as the nitrogen source gradually added to the bioreactor by a 250 ml separatory funnel during 24, 48 and 72 hr of fermentation. There was no nitrogen source initially in the culture medium.

A sample was withdrawn at the end of each run followed by centrifugation in order to analyze the produced biomass as well as secreted rhamnolipid in the medium. However, no sampling was done during the fermentation process to avoid variation in carbon to nitrogen molar ratio (C/N=10). All the experiments were performed in triplicate under the predetermined pH and temperature. These experimental data was used to describe the following proposed mathematical model.

The fed-batch mass balance equations are a bit more complicated than the batch ones. The equations of biomass growth, substrates uptake and rhamnolipid production are given as (10) to (15) for constant feed fed-batch fermentation [19].

$$\frac{dx_a}{dt} = \mu . x_a - x_a . D - k_d . x_a \quad (10)$$

$$\frac{d(x_i)}{dt} = -x_i . D + 0.2\, k_d . x_a \quad (11)$$

$$\frac{d(C_n)}{dt} = D(C_{n_i} - C_n) - \left(\frac{\mu}{Y\,x/n} + \frac{q_p}{Y\,p/n}\right)x \quad (12)$$

$$\frac{d(C_g)}{dt} = -D . C_g - \left(\frac{\mu}{Y\,x/g} + \frac{q_p}{Y\,p/g}\right)x \quad (13)$$

$$\frac{d(P)}{dt} = -P . D + q_p . x \quad (14)$$

$$x_{Tot} = x_a + x_i \quad (15)$$

where D is dilution rate (hr^{-1}), C_{n_i} is inlet sodium nitrate concentration (g l^{-1}), q_p is specific production rate (hr^{-1}), $Y_{p/n}$ and $Y_{p/g}$ are product to sodium nitrate and product to glycerol yield (g g^{-1}) respectively and P is rhamnolipid concentration in bioreactor (g rhamnose l^{-1}). Finite difference method under MATLAB programming software was also employed to predict the substrate, biomass and rhamnolipid concentrations during the nitrogen limiting fed-batch fermentation process.

Equation (11) is similarly explained as of (5). It was assumed that the term $q_p/Y_{p/n}$ in (12) was negligible since there was no nitrogen in the rhamnolipid structure [1]. Value for $Y_{p/g}$ in (13) was achieved experimentally by $Y_{x/g}/\,Y_{x/p}$ as explained in the following sections.

It should be noted that only the final concentrations of predicted biomass and rhamnolipid were comparable with the experimental values.

III. Results and Discussion

The accuracy of the prediction made by a proposed model mainly depends on the parameters used in it. Some of these parameters are calculated based on experimental data while estimating others are more complicated and needs to be validated by fitting the model on experimental data. In this study, as it was described before, it has been attempted to achieve the parameters $\mu_m, K_g, K_n, Y_{x/g}$, k_d and $Y_{p/g}$ experimentally or base on a simplified batch modeling while $Y_{x/n}$ and q_p achieved during a fed-batch model validation by experimental data. Finally, the ability of these parameters for a good prediction of the process behavior was assessed by performing verification experiments under different conditions. If the errors are small enough, the proposed fed-batch model is introduced as a reliable model for prediction of *P. aeruginosa* growth and rhamnolipid production on glycerol under nitrogen limiting fed-batch fermentation. This procedure is discussed in details in the following sections.

A. Unknown Parameters Calculation

As previously mentioned, values for μ_m, K_g and K_n can be estimated by fitting the experimental data of specific growth rates at different substrate concentrations (Table I) using the dual-parallel substrate Monod equation, (1) as the other models are not reliable to fit the experimental data (unpublished data).

Different glycerol concentrations (in the range of 0.1 to 30 g l^{-1}, C/N=10) were provided to calculate the specific growth rates according to (3). The relevant results are shown in Table I.

The dual-parallel substrate Monod equation well fitted these data with significantly high value of correlation of coefficient (R^2 =0.999) which indicated a reliable estimation of the kinetic parameters as were tabulated in Table II. As it is shown, the values of 0.691 hr^{-1}, 0.073 and 0.244 g l^{-1} were achieved for μ_m, K_g and K_n, respectively.

TABLE I
THE SPECIFIC GROWTH RATE VALUES UNDER BATCH FERMENTATIONS ON INITIAL GLYCEROL CONCENTRATIONS IN THE RANGE OF 0.1 TO 30 G L^{-1} (C/N=10). INITIAL INOCULUM CONCENTRATION = 0.22 G L^{-1} DRY WEIGHT

Run	C_g (g l^{-1})	C_n(g l^{-1})	$X_{1.5}$ (g l^{-1})[a]	μ(hr^{-1})
1	0.1	0.028	0.2513±0.0122	0.0972
2	0.5	0.138	0.3013±0.0135	0.2182
3	1	0.277	0.3630±0.0131	0.3424
4	1.5	0.415	0.4048±0.0137	0.4151
5	2	0.554	0.4342±0.0134	0.4618
6	4	1.108	0.4984±0.0146	0.5537
7	8	2.215	0.5479±0.0129	0.6168
8	11	3.046	0.5630±0.0168	0.6350
9	14	3.876	0.5726±0.0162	0.6462
10	18	4.984	0.5807±0.0163	0.6556
11	22	6.091	0.5862±0.0160	0.6619
12	26	7.199	0.5898±0.0147	0.6660
13	30	8.306	0.5932±0.0159	0.6698

[a] The mean biomass concentration after 1.5 hr of incubation± S.D., replicates=3.

TABLE II
THE MAIN PARAMETERS ESTIMATED UNDER BATCH AND FED-BATCH FERMENTATIONS

parameter	Value		Unit
	Batch	Fed-batch	
μ_m	0.691	-	hr^{-1}
K_g	0.073	-	g l^{-1}
K_n	0.244	-	g l^{-1}
k_d	0.152	-	hr^{-1}
$Y_{x/g}$	0.82	-	g g^{-1}
$Y_{x/n}$	4.73	1.16	g g^{-1}
$Y_{p/g}$	0.25	0.54	g g^{-1}
$q_{p,20}$[a]	-	0.038	hr^{-1}
$q_{p,60}$[b]	-	0.024	hr^{-1}

[a] The specific production rate at glycerol concentration of 20 g l^{-1}
[b] The specific production rate at glycerol concentration of 60 g l^{-1}

The smaller value for K_g than K_n suggests more tendency of *P. aeruginosa* to consume the carbon source than the nitrogen source. Among the similar works, Kim et al. achieved the values 0.14, 0.3 and 0.3 hr^{-1} for the maximum specific growth rate of *P. aeruginosa* based to Monod, Haldane–Andrews and Aiba–Edwards models, respectively [21]. Beyenal et al. also quantified the μ_m and saturation constant as 0.29 hr^{-1} and 0.0269 g l^{-1}, respectively using dual-substrate model for *P. aeruginosa* growth on glucose in a chemostat [22]. In another research, the maximum specific growth rate and Monod saturation constant parameters were achieved 0.23 h^{-1} and 0.178 g l^{-1}, respectively for growth of *Pichia pastoris* on glycerol by applying chemostat cultivation [23]. Value for K_g was estimated 1.5 g l^{-1} by Hekmat et al. in order to model the batch synthesis of dihydroxyacetone from glycerol by *Gluconobacter oxydans* [24].

The greater value for μ_m (0.691 hr^{-1}) as well as a smaller value for K_g (0.073 g l^{-1}) in the present study comparing to the other works suggest a faster growth and more tendency to glycerol consumption by *P. aeruginosa* than the other strains reported.

A vast range of glycerol concentrations was considered for calculation of these three kinetic parameters. Mathematically, when high concentrations of carbon and nitrogen sources are used, the terms $(K_g + C_g)$ and $(K_n + C_n)$ in (3) become approximately equal to K_g and K_n. In this case, (3) is simplified to $\mu_m \sim \mu$ which means that the specific growth rate is not affected by variation in carbon and nitrogen sources concentrations. Therefore, those parameters which were obtained based on experiments under only high glycerol concentrations are not acceptable (data not shown). On the other hand, it was found that the substrate concentrations (at studied range) had no inhibitory effect on biomass growth since no reduction was observed in specific growth rate values by increasing initial substrate concentrations (Table I).

Searching for the best values for $k_d, Y_{x/g}$ and $Y_{x/n}$ within the predetermined ranges, gives the minimum AARE value between experimental and theoretical x_{Tot} for 0.152 hr^{-1}, 0.82 and 4.73 g g^{-1} values, respectively. The estimated $Y_{x/g}$ was directly applied to the fed-batch modeling since it was assumed that entire carbon source was available for microorganisms in nitrogen limiting conditions (just like the batch process). Similarly Li et al. found that there was no significantly difference between values for $Y_{x/s}$ (yield coefficient on glucose) under batch and fed-batch incubation of *Pseudomonas* [6]. However in case of $Y_{x/n}$, it is not a reasonable assumption since microorganisms are in stress of nitrogen deficiency and the nitrogen source is not completely available for the bacteria. Therefore, it was attempted to achieve the individual value for $Y_{x/n}$ by fitting the fed-batch model on some individual experimental runs. The value of 1.16 g g^{-1} was finally given for $Y_{x/n}$ which was similar to values achieved by other researchers [25], [26]. The values of 0.55 and 0.3 g g^{-1} were also achieved for the parameter $Y_{x/g}$ for batch incubation of microorganism on glycerol as the sole carbon source [23], [24]. As can be seen, the studied *P. aeruginosa* in this work suggests a higher growth yield on glycerol than the reported ones.

The specific production rate, q_p, was also adjusted simultaneously with $Y_{x/n}$ during fitting the fed-batch model by experimental data. Dunn et al. reported that in fermentation processes involving product formation, the specific production rate often depended on fermentation conditions, e.g., substrate concentration [19]. Therefore, this led to estimating two different values of 0.038 and 0.024 hr^{-1} for q_p at studied glycerol concentrations of 20 and 60 g l^{-1}, respectively. This parameter also depends on dilution rate so these values were achieved by taking the average between values obtained under different flow rates [27].

The last parameter, $Y_{p/g}$, was estimated experimentally by dividing $Y_{x/g}$ (0.82 g g^{-1}) over $Y_{x/p}$. The term in the denominator is equal to $\Delta x/\Delta p$ which is readily calculated using the experimental data. It was found that, the values of 0.25 and 0.54 g g^{-1} were individually assigned to $Y_{p/g}$ in batch and fed-batch fermentation, respectively. Lee et al. proposed the values of 0.68 and 0.75 g g^{-1} for this parameter under batch

and fed-batch production of rhamnolipid by *P. aeruginosa* on fish oil [28]. The other values of 0.141 and 0.134 g g^{-1} have been reported by Li et al. for the yield coefficient of phenazine-1-carboxylic acid production on glucose under batch and fed-batch incubation of *Pseudomonas* [6]. The values of 0.8 and 0.17 g g^{-1} have also been proposed for biosynthesis on glycerol [3], [24].

B. Evaluation of the Proposed Fed-Batch Model

1. Relationship between Specific Growth Rate and Limiting Substrate Concentration

According to literature, the specific growth rate of microorganism can be effectively controlled by limiting source concentration [2], [29], [30]. This issue was well depicted in Fig. 1 by the proposed fed-batch model. As it is clear, the specific growth rate of the bacteria (curve a) increased by initial nitrate accumulation in the medium (the initial peak of the curve b) but it gradually decreased with increasing the bacterial population in the bioreactor as long as they consumed the entire surrounding nitrate.

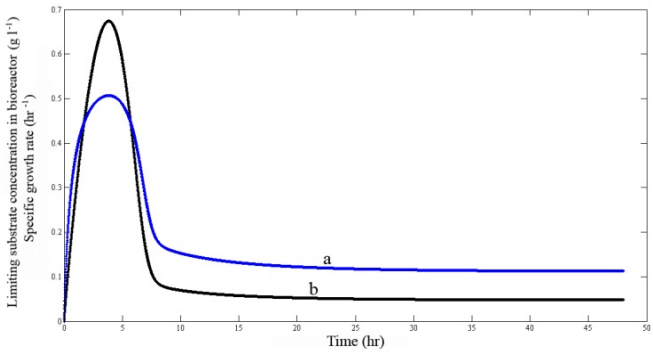

Fig. 1 Dependency of (a) specific growth rate of microorganism on (b) limiting substrate concentration (nitrogen source) in the bioreactor. Hypothetical parameters were considered

At this point, the entrance rate of nitrogen source equals to its consumption by microorganisms. It is a quasi-steady-state in limiting substrate concentration and specific growth rate [27]. Oberle and Sothmann previously showed the same trends for penicillin production using fed-batch fermentation as was indicated in Fig. 1 [31].

2. Describing the Model Using Experimental Data

The experimental data was achieved individually for fed-batch process by considering glycerol concentration (20 and 60 g l^{-1} with constant C/N=10) as well as feed flow rate (10.42, 5.21 and 3.47 ml hr^{-1}) as two main factors. All of the parameters obtained were placed in the model except for q_p and $Y_{x/n}$ which were simultaneously achieved by fitting the model on the experimental data.

It was found that, the value of 1.16 g g^{-1} was the best value fitted for $Y_{x/n}$ which lead to the most accurate prediction. However, $q_{p,60} = 0.024$ hr^{-1} and $q_{p,20} = 0.038$ hr^{-1} were also adjusted at glycerol concentrations of 60 and 20 g l^{-1}, respectively for the best prediction of rhamnolipid and biomass concentrations. According to Fig. 2, the predicted rhamnolipid values (1.10, 1.31 and 1.38 g l^{-1}) were highly compatible with the experimental data (1.22±0.07, 1.30±0.11 and 1.38±0.06 g l^{-1}, ±Standard Deviation) under 60 g l^{-1} glycerol concentration and predetermined feed flow rates of 10.42, 5.21 and 3.47 ml hr^{-1}, respectively. The latter value, which was achieved during 72 hr of fermentation, was almost four times higher than that for the batch process under the same conditions. However, the glycerol limiting fed-batch fermentation better stimulated the rhamnolipid production up to 12 times greater [18].

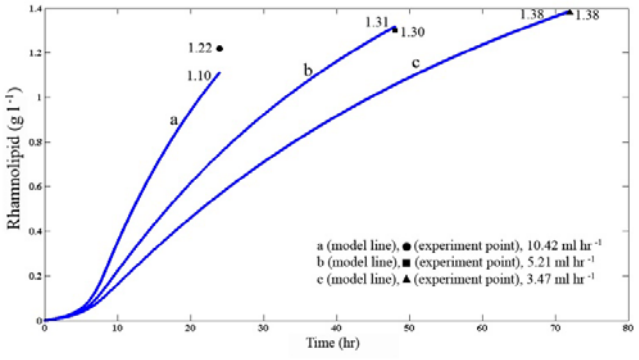

Fig. 2 Comparison between the final concentrations of experimental and predicted rhamnolipid at glycerol concentration of 60 g L-1 (C/N=10) and feed flow rates of 10.42 (a:●), 5.21 (b:■), and 3.47 (c:▲) ml hr-1

Therefore, it can be concluded that the fed-batch fermentation significantly increased the rhamnolipid production in comparison with the batch process, as was previously mentioned by Soberón-Chávez [2].

Fig. 3 also confirmed the ability of the proposed model to predict the rhamnolipid produced at glycerol concentration of 20 g l^{-1}. There are low errors of 9.43, 9.52 and 1.39% between the predicted (0.58, 0.69 and 0.73 g l^{-1}) and experimental (0.53±0.1, 0.63±0.08 and 0.72±0.14 g l^{-1}) values.

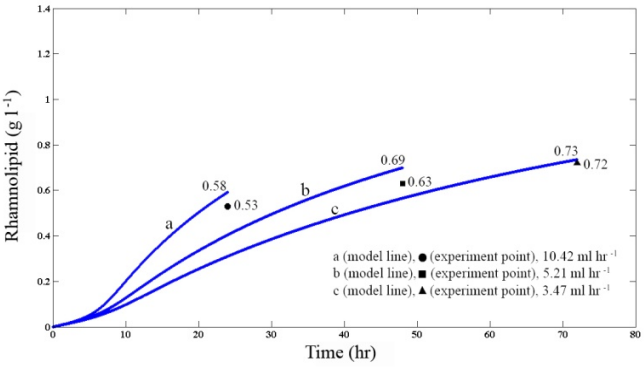

Fig. 3 Comparison between the final concentrations of experimental and predicted rhamnolipid at glycerol concentration of 20 g L^{-1} (C/N=10) and feed flow rates of 10.42 (a:●), 5.21 (b:■) and 3.47 (c:▲) ml hr^{-1}

As shown, the parameters used in the proposed model were successful enough to describe the experimental data with

maximum 10% error value. Both the model and the observed results suggested that the final rhamnolipid concentration decreased by increasing the feed flow rate (less feeding time). It can be due to the fact that more secretion of desired metabolite occurs within more process time. Although, it is clear from the slope of the lines in Figs. 2 and 3 that if the feed flow rates had not been limited by the feed volume (the experiments had been stopped at the same time), the results was different. It means that more rhamnolipid is produced by increasing the feed flow rate. In this case the working volume of the bioreactor becomes an important issue. It can be concluded that high feed flow rates control the specific growth rate in high levels which eventually leads to produce more rhamnolipid but the bioreactor will be overloaded faster. It is discussed in more detail at sensitivity analysis section.

As Figs. 4 and 5 illustrate, the model was not successful in prediction of total biomass concentration in the medium (active and dead) as was for rhamnolipids. The maximum error value was achieved 21% between the experimental and predicted biomass. However the trend which is observed in the total biomass concentrations has been previously reported by other researchers for variable volume fed-batch fermentations [31]–[34].

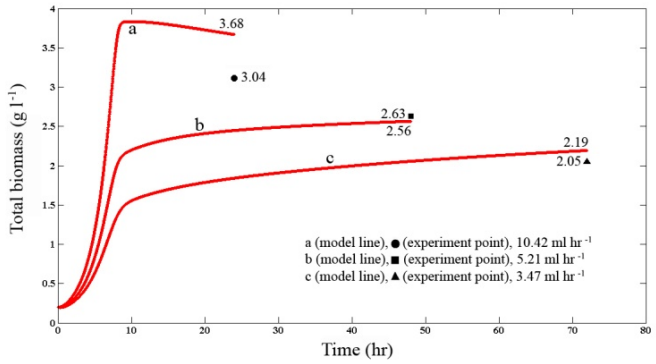

Fig. 4 Comparison between the final concentrations of experimental and predicted biomass at glycerol concentration of 60 g L^{-1} (C/N=10) and feed flow rates of 10.42 (a:●), 5.21 (b:■) and 3.47 (c:▲) ml hr^{-1}

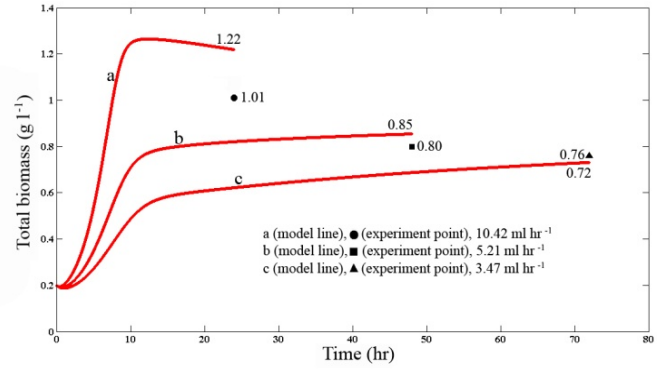

Fig. 5 Comparison between the final concentrations of experimental and predicted biomass at glycerol concentration of 20 g L^{-1} (C/N=10) and feed flow rates of 10.42 (a:●), 5.21 (b:■) and 3.47 (c:▲) ml hr^{-1}

On the other hand, increasing the initial glycerol concentration from 20 to 60 g l^{-1} increased the total biomass concentration almost three times.

The final slopes of the curves in Figs. 4 and 5 directly depend on the biomass accumulation in the medium as well as feed flow rate. The descending final slopes related to the highest flow rates (10.42 ml hr^{-1}) in Figs. 4 and 5; suggest that the feed flow rate prevailed on the biomass growth rate. The large error values (21%), which obtained under this flow rate, imply that the model is not suitable to predict the biomass concentration under high feed flow rates.

C. Verification Study

In order to confirm accuracy of the proposed model as well as the precision of the parameters estimated for prediction of biomass growth and rhamnolipid production, eight additional experiments were conducted randomly under different initial glycerol concentrations in the ranges of 10 to 100 g l^{-1} and a fixed nitrate feeding of 6.25 ml hr^{-1} during 48 hr of fermentation. All of these experiments were performed in duplicate.

The errors between the observed rhamnolipid and those predicted by the model were listed in Table III. As indicated, the maximum error value has been reported 9.67% except that at 100 g l^{-1} glycerol concentration which seemed unusual data. These data were tabulated based on two different q_p values ($q_{p,20}$ and $q_{p,60}$). The specified dash lines in the columns of Table III suggest that a bigger error value obtained under that respective q_p.

It can be seen that the adjusted value for $q_{p,20}$, 0.038 hr^{-1}, was only compatible up to 40 g l^{-1} glycerol concentrations (less than 10% error) while it was not a good estimation for higher glycerol concentrations.

TABLE III
THE FED-BATCH VERIFICATION EXPERIMENTS AT A FIXED FEED FLOW RATE OF 6.25 ML HR^{-1} AND INITIAL GLYCEROL CONCENTRATIONS IN THE RANGE OF 10 TO 100 G L^{-1} (C/N=10)

Initial glycerol conc. (g l^{-1})	Rhamnolipid production (g l^{-1})			
	experimental [a]	model		% Error
		$q_{p,20}$	$q_{p,60}$	
10	0.42±0.113	0.381	-	9.21
20	0.82±0.071	0.759	-	7.40
30	1.04±0.141	1.069	-	9.08
40	1.42±0.127	1.515	-	6.71
60	1.59±0.042	-	1.436	9.67
70	1.64±0.085	-	1.675	2.15
80	1.80±0.099	-	1.914	6.34
100	1.94±0.127	-	2.392	23.31

[a] Values are mean ± S.D. , replicates=2

By considering $q_{p,60} = 0.024$ hr^{-1}, the rhamnolipid prediction improved at higher glycerol concentrations up to 80 g l^{-1} with errors less than 10% (Table III). However, this value did not support higher glycerol concentrations (23% error at 100 g l^{-1} glycerol). A clear divergence was observed between the experimental and the predicted results at high glycerol concentrations that might be due to the negative impact of

concentrated nitrate on rhamnolipid production (C/N=10). As it has been previously cited, its production is more induced in the nitrogen deficiency conditions [2], [3].

As another point, the value of $q_{p,60}$ found to be less than $q_{p,20}$. Indeed, plotting the experimental rhamnolipids versus the initial glycerol concentrations is composed of two different slopes. The initial steeper slope at $q_{p,20}$ followed by a gentle slope at $q_{p,60}$.

It implies that the rhamnolipid production by *P. aeruginosa* is more sensitive at low glycerol concentrations.

The maximum error for prediction of the biomass was achieved 23% (data not shown). As previously mentioned, the model precision was lower for biomass prediction than for rhamnolipid. Moreover a linear relationship was also observed between the experimental biomass and initial glycerol concentrations. The same linear relationship has been also reported by Lee et al. when investigated the growth of *P. aeruginosa* on fish oil [28].

Therefore, it was found that the proposed model as well as the parameters estimated could be applied for prediction of rhamnolipid produced by *P. aeruginosa* on glycerol concentrations up to 80 g l^{-1} as the sole carbon source, C/N=10, and under nitrogen limiting fed-batch fermentation. However, the biomass prediction results are less satisfactory with maximum 23% error value.

D. Sensitivity Analysis

Sensitivity analysis was conducted in order to investigate the influences of controllable factors including feed flow rate, feed concentration and inoculum concentration on the final biomass and rhamnolipid concentrations. It can also be applied to optimize the process performance. It should be noted that, only one of the mentioned factors was simultaneously changed while the others were kept constant.

According to Fig. 6, depicted at initial glycerol concentration of 30 g l^{-1} and 72 hr of incubation, increasing the feed flow rate from 1.39 to 6.94 ml hr^{-1} intensified the initial peak of the limiting substrate concentration in the bioreactor.

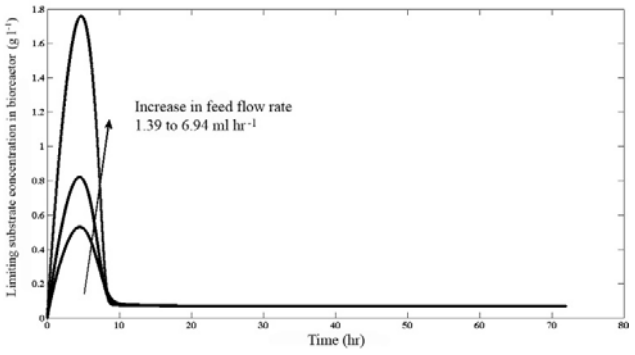

Fig. 6 The effect of increasing feed flow rate from 1.39 to 6.94 ml hr^{-1} on limiting substrate concentration in the bioreactor during 72 hr fermentation

It was due to exposing identical population of bacteria to more substrate which directly affects on the biomass growth and consequently rhamnolipid production, as illustrated in Figs. 7 (a) and (b). Biomass growth and rhamnolipid production increased from 0.6 to 1.5 g l^{-1} and 0.7 to 1.5 g l^{-1}, respectively.

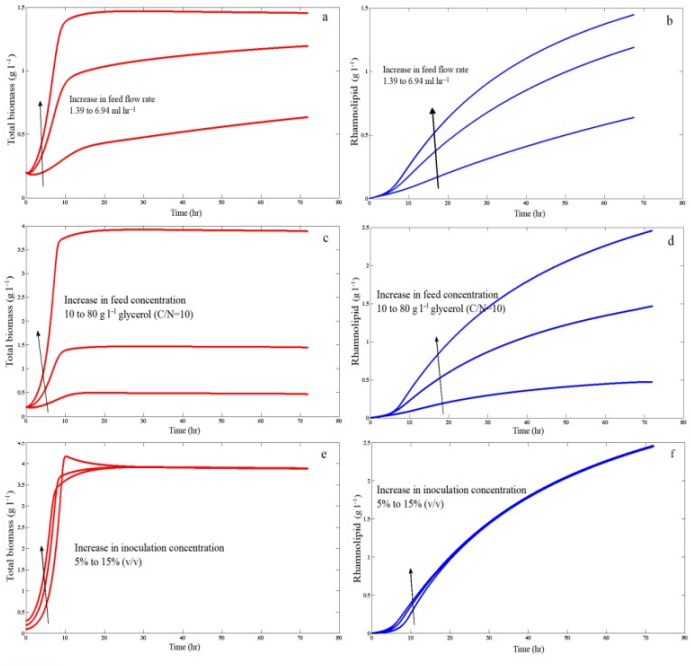

Fig. 7 (a) The effect of feed flow rate changes on the biomass and (b) rhamnolipid production under initial glycerol concentration of 30 g l^{-1} (C/N=10), inoculum concentration of 0.2 g l^{-1} and 72 hr fermentation time. (c) The effect of feed concentration changes on the biomass and (d) rhamnolipid production at a fixed feed flow rate of 6.94 ml hr-1, inoculum concentration of 0.2 g l^{-1} and 72 hr fermentation time. (e) The effect of inoculation concentration changes on the biomass and (f) rhamnolipid production at a fixed feed flow rate of 6.94 ml hr^{-1}, initial glycerol concentration of 80 g l^{-1} and 72 hr fermentation time

In case of increasing feed flow rate for more production, the substrate consumption will be more and faster which this is not economic in bioprocess engineering. Moreover the bioreactor will be quickly overloaded which is needed to be discharged regularly that this is time consuming as well as increases the contamination probability.

Limiting substrate concentration imposed the same effect on the biomass growth and rhamnolipid production. According to Figs. 7 (c) and (d), at a fixed feed flow rate of 6.94 ml hr^{-1} and 72 hr of incubation, increasing glycerol concentration from 10 to 80 g l^{-1} (C/N=10) enhanced the biomass and rhamnolipid concentrations to 4 and 2.5 g l^{-1} respectively. This direct relationship was previously demonstrated in Figs. 2 and 3 as well as Table III. Birol et al. also reported a direct relationship between initial glucose concentration in the feed and produced penicillin [33]. Figs. 7 (e) and (f) showed that the increase in initial inoculum concentrations from 0.1 to 0.3 g l^{-1} (5% to 15% v/v) had no effect on the final biomass and rhamnolipid concentrations.

However, it increased the initial rate of substrate consumption followed by faster achieving to the quasi-steady-state conditions in the bioreactor.

Therefore, the sensitivity analysis showed that the biomass and rhamnolipid production would be maximized up to 4 and 2.5 g l^{-1} respectively under feed flow rate of 6.94 ml hr^{-1}, 80 g l^{-1} glycerol concentration (C/N=10) and 72 hr of incubation. It also confirmed the critical role of substrate flow rate and substrate concentration on process performance. Both of these controllable factors can be served to produce high rhamnolipid concentration by *Pseudomonas aeruginosa* ATCC 53752, however selection of each is highly depend on either the bioreactor volume or economic limitations.

IV. Conclusion

Rhamnolipid is an important and widely used metabolite which is produced during the stationary phase of growth. Among various production methods, fed-batch cultivation is the most effective process to achieve high productivity of rhamnolipid. An efficient process modelling helps to optimize the process without the need for additional experiments which are expensive and time consuming. In this study a nitrogen limiting fed-batch process was mathematically modeled in order to effectively predict the biomass growth and rhamnolipid production by *Pseudomonas aeruginosa* ATCC 53752. Various verification experiments were performed to assess the accuracy of the predictions by the proposed model.

It was found that the rhamnolipid prediction results were quite satisfactory with less than 10% error. This also confirmed the accuracy of the parameters obtained. There was only a limitation for the use of q_p under different ranges of glycerol concentrations. The adjusted value of q_p =0.038 hr^{-1} well responded for up to 40 g l^{-1} glycerol while the value of q_p =0.024 hr^{-1} was more appropriate for higher levels up to 80 g l^{-1}. In addition, it was found that rhamnolipid production by the studied strain was more sensitive to lower substrate concentrations than the higher levels. In contrast of rhamnolipid prediction, biomass prediction results were not satisfactory since the prediction errors increased up to 23%. Furthermore a simple optimization was performed using sensitive analysis, after ensuring the model precision especially for rhamnolipid production. This indicated that the maximum biomass and rhamnolipid concentration were achieved 4 and 2.5 g l^{-1} under feed flow rate of 6.94 ml hr^{-1}, glycerol concentration of 80 g l^{-1} (C/N=10), and 72 hr incubation.

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