Int J Clin Exp Med 2017;10(4):6468-6474
www.ijcem.com /ISSN:1940-5901/IJCEM0041344

Original Article

A kinetic model for phenazine-1-carboxylic acid production by *pseudomonas* sp. M18G

Chao Cheng[1], Guijun Ma[1], Li Deng[3], Rongxiu Li[1,2]

[1]*State Key Laboratory of Microbial Metabolism, School of Life Sciences & Biotechnology, Shanghai Jiaotong University, Shanghai, China;* [2]*Engineering Research Center of Cell & Therapeutic Antibody, Ministry of Education, Shanghai, China;* [3]*Shanghai HyCharm Inc., Shanghai, China*

Received August 10, 2016; Accepted February 4, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: The development of optimal bacterial strains for industrial scale of fermentation would benefit from a quantitative model of rate of gene expression from DNA to RNA, from DNA to protein, and ultimately from DNA to the metabolites. In this study, we developed such a model for phenazine-1-carboxylic acid (PCA) biosynthesis in Pseudomonas sp. strain M18G. The PCA biosynthesis is controlled by anoperon phzABCDEFG. Two variables were modeled during fermentation: the rate of phzCmRNA synthesis and the rate of PCA production. A kinetic model was established based on Logistic and Luedeking-Piret equations. Our data demonstrated that the kinetic model provides a good description of temporal variations of biomass, product and mRNA levels in PCA fermentation process. Based on estimated kinetic parameters, this model was used to improve the PCA production in Pseudomonas sp. strain M18G, and monitor the level of phzC mRNA, which act as a reference index in optimization of engineering bacteria. In addition, the model can be applied for monitoring internal state of cells in the process of large scales fermentation.

Keywords: Fermentation, logistic equation, Luedeking-Piret equations, kinetic model, *pseudomonas* sp. M18G, phenazine-1-carboxylic acid, *phz*C mRNA

Introduction

Microbial fermentation is controlled by genetic information from the transcription of DNA into mRNA, and translation to protein, and then enzyme-catalyzed biosynthesis to final metabolic products, which is also called “the central dogma” [1, 2]. The fermentation yield depends on the regulation mechanisms at various levels of the central dogma inside microbial cells as well as the supply of building blocks of the products [3, 4]. Compared to the conventional intermediate products, the “central dogma” describes an information transmission rather than a material transport process. It is crucial to elucidate the intracellular biochemical process for the optimization of product yield and improvement of economic efficiency [5, 6].

Pseudomonas sp. strain M18 resides in the rhizosphere of sweetmelon, and is the only strain known so far to produce bothphenazine-1-carboxylicacid (PCA) and pyoluteorin [7, 8]. For modeling PCA production, we used a *Gac*A-deficient mutant strain of M18, M18G, in which the pyoluteorin production was inhibited completely, but PCA production is increased over 30-fold [9]. *Phz*C is one part of the seven-gene operon *phz*ABCDEFG, responsible for the synthesis of PCA in *Pseudomonas* species [10]. Therefore, we speculated that the enhancement of the *phz*C transcriptional level could also increase the production of PCA.

The conventional methods of optimizing fermentation have been focusing on developing a mutant strain and identifying optimal cultivation conditions, e.g., pH, temperature and dissolved oxygen and nutrition, which indirectly changed the intracellular processes. These conventional approaches suffer from low efficiency and time consuming. Traditional parameters are external display of “central dogma” [*11*]; hence, monitoring of the “central dogma” can directly reflect the intracellular biochemical process and fermentation process. It is also

beneficial to identify key genes and optimize engineering strain.

In fact, the central dogma as well as the key regulatory factors will provide more useful information and optimization of the fermentation process for the quantitative relationship between product yield and kinetic model [12]. Studies have pointed out that the relationship between transcription level and protein, which have found that the steady-state protein level is directly proportional to mRNA level [13]. However, these descriptions of the relationship in central dogma have been improved although were still non-quantitative [14, 15].

Transcription is the first step of gene expression, in which a particular segment of DNA is copied into mRNA. The mRNA will in turn serve as a template for the translation of protease, which can be used to catalyze the metabolites synthesis, and to determine the formation rate together with other mechanisms. Our laboratory has recently developed a method based on molecular beacon, which can rapidly detect the concentration of *phz*C mRNA in the process of PCA fermentation [16]. Herein, we monitored the biomass, *phz*C mRNA and PCA production in *Pseudomonas* sp. strain M18G fermentation, and a kinetic model was developed to demonstrate the relevance among them with time variation. The correlation between mRNA and PCA in the fermentation process was consistent with previous work [14].

Materials and methods

Strain and culture conditions

The strain used in this study was *Pseudomonas* sp. strain M18G stored in our laboratory. It was maintained in glycerol (20%, by volume) and stored at -70°C. For seed preparation, single-colony isolates of *Pseudomonas* sp. M18G were inoculated into 50 mL of King's B medium in 250 mL flasks and incubated for 10 h at 28°C with shaking at 165 rpm on a rotary shaker. The King's B medium containing 20 g peptone, 15 g agar, 0.392 g K_2HPO_4, 0.732 g $MgSO_4$, and 15 mL glycerol per liter, pH 7.5 [17].

In fermentation experiment, the seed culture was then transferred into and incubated at (5% volume) 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium. The inoculated flasks were kept on a rotary shaker at 165 rpm at 28°C. The fermentation medium containing 33 g soybean powder, 11 g soya peptone, 12 g glucose, 4 mL glycerol, and 13 mL ethanol per liter, the initial pH value of the media had been adjusted to 7.8-8.0 prior to autoclaving.

Analytical methods

Biomass was measured as dry cell weight [18]. PCA extraction and detection was done according to the reference [19]. Detection and quantitative analysis of *phz*C mRNA were performed by MB hybridization with an oligodeoxynucleotide probe [16].

Kinetic models

The kinetic models for PCA fermentation included temporal variations of biomass (C, dry cell weight, $g{\cdot}L^{-1}$), product (P, PCA yield, $g{\cdot}L^{-1}$), and temporal variations of mRNA (Rm, mRNA, unit L^{-1}).

Microbial growth: the logistic equation

Some features of PCA fermentation process could be concluded from previous work. Similar biomass values were detected in the stationary phase of batch fermentations with different initial glucose concentrations, which indicated that the M18G fermentation process does not follow the classical kinetic model of substrate-limited biomass growth proposed by Monod [20]. But the logistic equation that had been widely applied as a substrate-independent model for population growth could be used as an alternative empirical function [21]. The Logistic equation can be described as follows:

$$\frac{dC}{dt} = \mu_m C \left(1 - \frac{C}{C_m}\right) \quad (1)$$

Where μm is the maximum specific growth rate, h^{-1}; Cm is the maximum attainable biomass concentration, gL^{-1}.

Production formation: the Luedeking-Piret equation

The kinetics of product formation was based on the Luedeking-Piret's equation [22]. The classical Luedeking-Piret equation can be described as follows:

Table 1. Kinetic models and estimation of model parameters

Model	Parameter	Value
$\frac{dC}{dt} = \mu_m C (1 - \frac{C}{C_m})$	μ_m, h^{-1}	0.120
	C_m, g L^{-1}	6.000
$\frac{dP}{dt} = \alpha \frac{dC}{dt} + \beta C$	α, g L^{-1}	0.221
	β, g L^{-1} h^{-1}	-0.0002
$Cm = \alpha_m \frac{dC}{d(t - t0)} + \beta_m C$	α_m, g L^{-1}	32.366
	β_m, g L^{-1} h^{-1}	0.041
	t0, h	8

μm = maximum specific growth rate; C = biomass concentration; Cm = maximum biomass concentration; dP/dt = production rate; α = growth associated product formation coefficient; β = non-growth associated product formation coefficient; *Cm* = mRNA concentration; t = fermentation time.

$$\frac{dP}{dt} = \alpha \frac{dC}{dt} + \beta C \quad (2)$$

Where α (g·L^{-1}) and β (g·L^{-1} h^{-1}) are the product formation constants. While the β value represents the non-growth-associated process, the larger the α value, the more growth-associated the fermentation process is.

mRNA transcription: the modified Luedeking-Piret kinetic equation

The kinetics models for PCA fermentation include cell growth kinetic model, product formation kinetic model, and substrate consumption kinetic model. In general, the Luedeking-Piret equation is used to describe the product formation. If the mRNA is considered as an intermediate product, this kinetic model can be expressed as follows:

$$\frac{dR_m}{dt} = \alpha_m \frac{dC}{d(t)} + \beta_m C \quad (3)$$

Where *Rm* is the concentration of mRNA (g L^{-1}), α_m and β_m are empirical constants that may vary with fermentation conditions (temperature, pH, etc.). However, it was found that the initial time of mRNA transcription will be in advance or delay when compared with product formation. Using the time parameter and substituting Equation 3 into Equation 4, integrating yielded the following equation:

$$\frac{dR_m}{dt} = \alpha_m \frac{dC}{d(t - t0)} + \beta_m C \quad (4)$$

Where *t0* is the initial time of mRNA transcription. In addition, Equation 4 cannot be directly used to calculate the accumulation of mRNA since the mRNA degradation, which is a key process to control the steady-state of gene expression. The analysis of mRNA degradation and quantitative of a single gene indicated that half-life of most mRNAs were shorter than products [23]. Therefore, the transient expression of mRNA is considered as the accumulation amount. Taking both transcription and degradation of mRNA into consideration, Equation 4 is rearranged as:

$$Cm = \alpha_m \frac{dC}{d(t - t0)} + \beta_m C \quad (5)$$

Where *Cm* is the concentration of mRNA(g L^{-1}).

Parameters estimation

Estimates for model parameters were obtained with the MATLAB software using the experimental data from fermentation. These values were evaluated by fitting the mathematical models to the experimental data, using the least-squares method, so as to minimize the sum of the squares of the deviations between the experimental measurements and the model predications.

Results and discussion

M18G growth curve

The Monod equation has been widely applied to describe microbial growth. However, this model is generally more suitable for describing substrate-limited growth with low cell populations, but cannot fit well the processes of batch fermentation, in our case. The logistic rate equation could describe well the inhibition of biomass on growth, which exists in many batch fermentations, and therefore chosen to model the dynamics of PCA fermentation in M18G.

By fitting the experimental data to Equation 1, the values of the parameters of biomass concentration were estimated with the MATLAB software and found to be the following: μ_m = 0.12 h^{-1}; C_0 = 0.2 g L^{-1}; and Cm = 6.00 g L^{-1} (**Table 1**). Observing from **Figure 1**, mRNA production via the fermentation by the *Pseudomonas* sp. strain M18G in batch cultures followed a typical "S" curve, in accordance with models of the growth of other microorganisms.

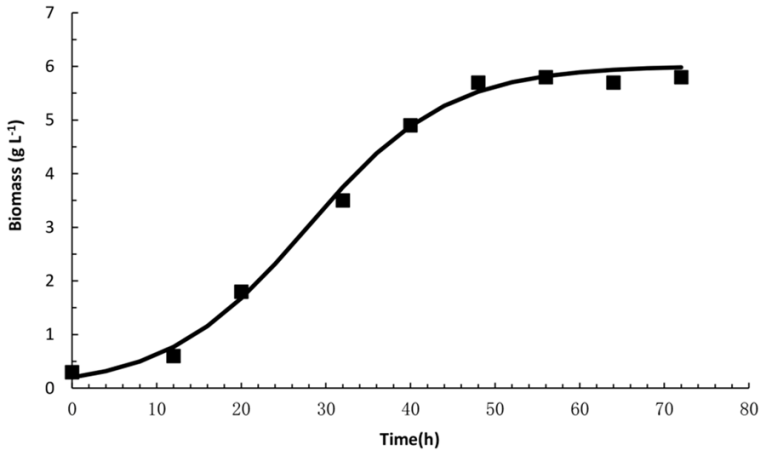

Figure 1. Time courses of experimental (filled square) and calculated (line) biomass by *Pseudomonas* sp. M18G in batch fermentation.

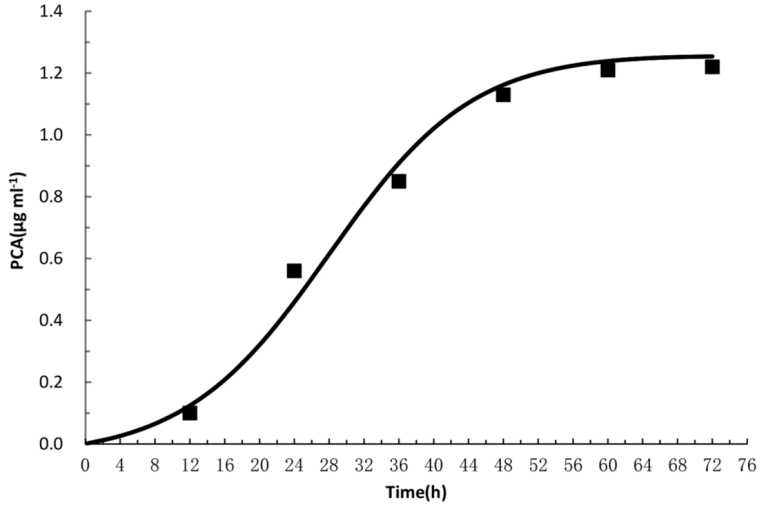

Figure 2. Time profiles of experimental (filled square) and calculated (line) PCA production by *Pseudomonas* sp. M18G in batch fermentation.

After a short lag phase, cells entered the exponential growth phase when the production of *phz*CmRNA was also started. The experimental data fitted well to the model with correlation coefficient (r^2) of model fitting was 0.994.

Production formation

By fitting the experimental data to Equation 2, the coefficient values of the product formation and the non-growth associated product formation (α = 0.221 g L^{-1}, β = -0.0002 g L^{-1} h^{-1}) suggested that the model was able to describe the PCA formation as the fermentation follows S curve. The predicated values obtained from the kinetic model for PCA accumulation are listed in **Table 1**. The experimental data fitted well to the model with correlation coefficient (r^2) of 0.991. The PCA production by *Pseudomonas* sp. M18G was increased proportionally to the concentration of the biomass in the exponential growth phase, and continued to increase even during the stationary phases (**Figure 2**). This observation illustrates that a kinetic of PCA production follows the classical Luedeking-Piret model. In batch fermentation, the PCA formation rate showed a similar trend with the growth rate of *Pseudomonas* sp. M18G, and the predicted time to reach maximum specific growth rate and the maximum volumetric productivity of PCA are the same (**Figure 3**). This model could thus be applied feasibly to the description of the batch fermentation of PCA.

Correlation between phzC mRNA and PCA production

Based on Luedeking-Piret model, the time dependent *phzC* mRNA production was calculated. Like PCA, the formation of *phzC* mRNA by *Pseudomonas* sp. M18G follows the product formation pattern. Subsequently, the modified Luedeking-Piret equations depicting the level of *phzC* mRNA were developed, which added with time dependent parameters and modified with product formation rate into the product concentration. Thus, the estimated model was able to fit experimental observations very satisfactorily.

The coefficients of *phzC* mRNA formation in *Pseudomonas* sp. M18G (Equation 5) were estimated respectively in the same manner and the values are shown in **Table 1**. The correlation coefficient (r^2) of the model was 0.954. A comparison of the calculated values with the experimental data was given in **Figure 4**. Fed-batch

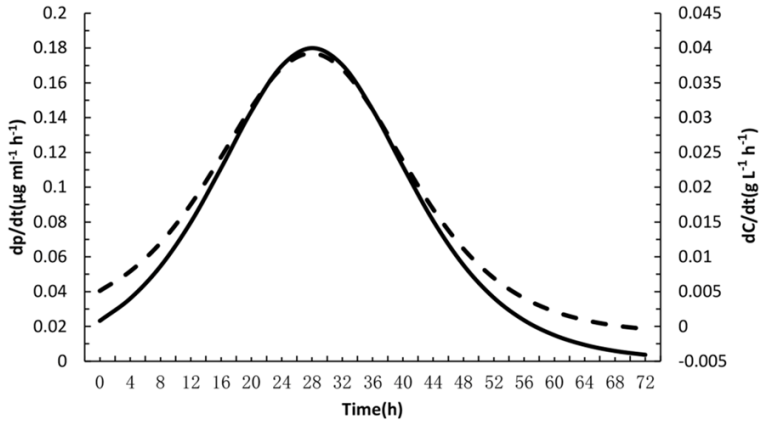

Figure 3. Time profiles of cell specific growth rate (full line) and PCA production rate (dashed line).

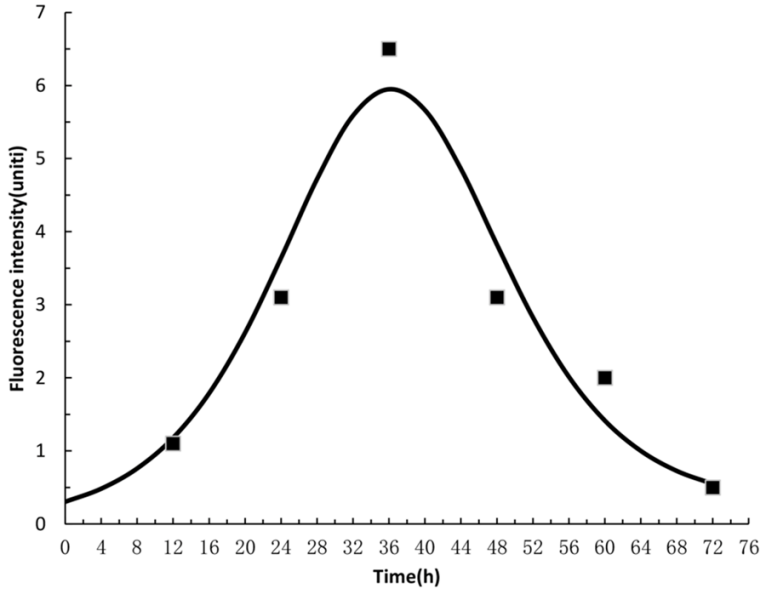

Figure 4. Comparison of calculated (line) with the experimental (filled square) *phz*C mRNA concentrations.

culture was performed with the initial cell concentration of 0.2 g·L^{-1}, the *phzC* mRNA was predicted to reach a maximum value of 0.595 U·mL^{-1} at 36 h of fermentation. The data showed that this model could satisfactorily explain the kinetics of the fermentation process in terms of *phzC* mRNA production in *Pseudomonas* sp. M18G.

By measuring the levels of *phzC* mRNA, we also examined the correlations between *phzC* mRNA and PCA. *phzC* gene is one component of the PCA biosynthesis operon *phzABCDEFG*, so the transcription levels of *phzC* were directly correlated with the yield of PCA. During the fed-batch culture, we found that the *phzC* mRNA levels were linearly correlated with the PCA level (**Figure 5**). The concentration of *phzC* mRNA was linearly proportional to the synthesis rate of PCA in the early-log phase and stationary phase, indicating that there was no other factor to control the formation of the product at this time. Relationship between PCA synthesis rate and *phzC* mRNA in the mid-log phase behavior in a non-linear pattern, we believed that this may be due to the rapid growth of cell and product inhibition.

At the turning point in batch fermentation process (28 h), both of PCA synthesis and cell growth reached the maximum. However, the PCA synthesis rate decreased with the further rising of *phz*C mRNA in the late-log phase, which indicated that there exist some negative regulations or other bottle necks in this period. This proportionality reflected that transcription and translation processes are coupled.

Conclusion

Gene expression is fundamental in all the life process, and has been intensively investigated. However, very little is known about the kinetics of bacterial gene expression during fermentation process. Compared to the monitoring of cultural parameters, quantitative description of the transcription process provides more useful information for genetic improvement of industrial strain and can also provide references to designing monitor for early signal in industrial fermentation processes. In this study, PCA fermentation based on a simple medium by *Pseudomonas* sp. strain M18G was investigated. The bacterial growth follows the logistical equation, and the PCA formation conforms to

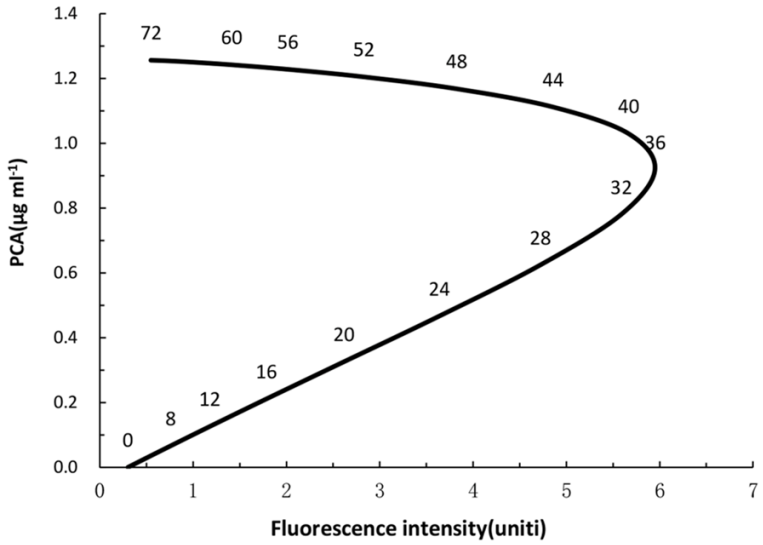

Figure 5. Relationship between *phz*C mRNA formed and PCA produced by *Pseudomonas* sp. M18G in batch fermentation.

the Luedeking-Piret model. Generally, one would assume that the amount of the intermediate mRNA would influence the amount of the end product. However, the half-life of mRNA in viable bacterial cells reported to be in the range of minutes, and is shorter than that of most products, which means that the transient expression of mRNA can be considered as the accumulation. The Luedeking-Piret model was unable to fit experimental observations very satisfactorily, so we developed a modified Luedeking-Piret equation to describe the mRNA, which added with time dependent parameters and modified with product formation rate into the product concentration. Therefore, the model can be applied for posterior optimization and controller process design. The results predicted from the model were in good agreement with the experimental observations. Therefore, the fermentation process of *Pseudomonas* sp. M18G was described satisfactorily using differential equations, and the models also could explain the correlation between mRNA and products. The models proposed here would be helpful to develop and optimize the industrial bioprocess to improve productivity and efficiency. The parameters described in the models can be used for improving PCA production by *Pseudomonas* sp. M18G and optimizing engineering bacteria.

Disclosure of conflict of interest

None.

Address correspondence to: Rongxiu Li, State Key Laboratory of Microbial Metabolism, School of Life Sciences & Biotechnology, Shanghai Jiaotong University, Shanghai HyCharm Inc., 1 Fute Street East, Bldg. 458, Rm. 518, Shanghai 200131, China. Tel: 86-021-34205517; Fax: 86-021-34205517; E-mail: rxli@sjtu.edu.cn

References

[1] Crick F. Central dogma of molecular biology. Nature 1970; 227: 561-563.

[2] Li GW and Xie XS. Central dogma at the single-molecule level in living cells. Nature 2011; 475: 308-315.

[3] Du X, Li Y, Zhou Q and Xu Y. Regulation of gene expression in pseudomonas aeruginosa M18 by phenazine-1-carboxylic acid. Appl Microbiol Biotechnol 2015; 99: 813-825.

[4] Zhou L, Jiang HX, Sun S, Yang DD, Jin KM, Zhang W and He YW. Biotechnological potential of a rhizosphere pseudomonas aeruginosa strain producing phenazine-1-carboxylic acid and phenazine-1-carboxamide. World J Microbiol Biotechnol 2016; 32: 1-12.

[5] An H, Scopes R, Rodriguez M, Keshav K and Ingram L. Gel electrophoretic analysis of zymomonas mobilis glycolytic and fermentative enzymes: identification of alcohol dehydrogenase II as a stress protein. J Bacteriol 1991; 173: 5975-5982.

[6] Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Chen F, Foston M, Ragauskas A and Bouton J. Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci U S A 2011; 108: 3803-3808.

[7] Hu HB, Xu YQ, Chen F, Zhang XH and HUR BK. Isolation and characterization of a new fluorescent pseudomonas strain that produces both phenazine 1-carboxylic acid and pyoluteorin. J Microbiol Biotechnol 2005; 15: 86-90.

[8] Yan A, Huang X, Liu H, Dong D, Zhang D, Zhang X and Xu Y. An rhl-like quorum-sensing system negatively regulates pyoluteorin production in pseudomonas sp. M18. Microbiology 2007; 153: 16-28.

[9] Ge Y, Huang X, Wang S, Zhang X and Xu Y. Phenazine-1-carboxylic acid is negatively regulated and pyoluteorin positively regulated by gacA in pseudomonas sp. M18. FEMS Microbiol Lett 2004; 237: 41-47.

[10] Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G and Thomashow LS. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from pseudomonas aeruginosa PA01. J Bacteriol 2001; 183: 6454-6465.

[11] Xiong ZQ, Tu XR and Tu GQ. Optimization of medium composition for actinomycin X2 production by streptomyces spp JAU4234 using response surface methodology. J Ind Microbiol Biotechnol 2008; 35: 729-734.

[12] LaRossa RA. Making metabolism accessible and meaningful: is the definition of a central metabolic dogma within reach? Biotechnol Lett 2015; 37: 741-751.

[13] Koonin EV. Why the central dogma: on the nature of the great biological exclusion principle. Biol Direct 2015; 10: 1-5.

[14] Golding I, Paulsson J, Zawilski SM and Cox EC. Real-time kinetics of gene activity in individual bacteria. Cell 2005; 123: 1025-1036.

[15] Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A and Xie XS. Quantifying E. Coli proteome and transcriptome with single-molecule sensitivity in single cells. Science 2010; 329: 533-538.

[16] Dong D, Pang Y, Gao Q, Huang X, Xu Y and Li R. Rapid monitoring of mRNA levels with a molecular beacon during microbial fermentation. J Biotechnol 2010; 145: 310-316.

[17] Sambrook J, Fritsch EF and Maniatis T. *Molecular cloning*. Vol. 2. New York: cold spring harbor laboratory press; 1989.

[18] He L, Xu YQ and Zhang XH. Medium factor optimization and fermentation kinetics for phenazine-1-carboxylic acid production by pseudomonas sp. M18G. Biotechnol Bioeng 2008; 100: 250-259.

[19] Li Y, Jiang H, Xu Y and Zhang X. Optimization of nutrient components for enhanced phenazine-1-carboxylic acid production by gacA-inactivated Pseudomonas sp. M18G using response surface method. Appl Microbiol Biotechnol 2008; 77: 1207-1217.

[20] Monod J. The growth of bacterial cultures. Annual Reviews in Microbiology 1949; 3: 371-394.

[21] Weiss RM and Ollis DF. Extracellular microbial polysaccharides. I. Substrate, biomass, and product kinetic equations for batch xanthan gum fermentation. Biotechnol Bioeng 1980; 22: 859-873.

[22] Luedeking R and Piret EL. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Reprinted from Journal of Biochemical and Microbiological technology and engineering Vol. I, No. 4. Pages 393-412 (1959). Biotechnol Bioeng 2000; 67: 636-44.

[23] Schoenberg DR and Maquat LE. Regulation of cytoplasmic mRNA decay. Nat Rev Genet 2012; 13: 246-259.