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Parameter optimization of pyoverdine content and growth kinetics on *Pseudomonas fluorescens* pf-10 strain in iron deficient liquid state media

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Iron nutrition in bacteria presents a fundamental challenge due to its extremely low solubility in aerobic environments at moderate pH. For optimal growth, *Pseudomonas* bacteria necessitate about 10 e^{-6} to 10 e^{-7} M of bioavailable iron and therefore solve an iron supply problem for survival by synthesizing and exporting a low-molecular-weight compound called siderophore. The latter has a high iron binding capability from the bacterial cell, and then importing it once bound to iron. A system of nonlinear ordinary differential equations has been studied and a parameter identification study conducted on unknown parameters along with a sensitivity analysis to determine key factors that contribute most to the variation in model outputs (experimental observations). The model has been quantitatively validated against population size count and pyoverdine content measurements obtained from *Pseudomonas fluorescens* pf-10 strain, the model solution can be used as an indirect experimental tool for developing practical criteria in plant growth-promoting rhizobacteria (PGPR) selections which are known to provide effective rhizosphere colonization, together with accurate predictions of iron depletion in the system over time.

Key words: *P. fluorescens* Pf-10, pyoverdine, Iron chelating, model calibration, sensitivity analysis.

INTRODUCTION

Iron availability is low in many soils; hence, microorganisms have evolved mechanisms to acquire this nutrient by, altering the chemical conditions that affect its solubility. Microorganisms respond to Fe deficiency by production and release of specific chelators. In the rhizosphere, the demand for Fe results in competition between plants and microorganisms with the latter being more competitive due to their ability to decompose plant-derived chelators and their proximity to the root surface (Marschner et al., 2011). These chelates, which were designated earlier as siderochromes, sideramines, and sideromycins, are now conveniently

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termed siderophores, technically defined as the ferric iron specific, low molecular weight (<1500) compounds, which solubilize and transport iron to the cell.

Among siderophores, pyoverdine is a water-soluble yellow-green fluorescent (under UV light) pigment which is composed of approximately 40 different structures established from various strains. Besides that, more than 70 different pyoverdines structures are expected from isoelectric focusing and iron uptake patterns determination studies (Emery, 1977; Meyer, 2000).

Pseudomonas strains which are known to be natural siderophore producers, have been isolated from different habitats around worldwide. Most species are saprophytes that are commonly found in water, soil and plant surfaces (Haas and Défago, 2005; Lugtenberg et al., 2001). Among Pseudomonads, 23 species are pathogenic to plants, including *Pseudomonas syringae* with 36 pathovars affecting different plants. In addition, 16 species are associated with diseases in humans and animals (Peix et al., 2009).

Plant-beneficial pseudomonads also called plant growth-promoting rhizobacteria (PGPR) release a remarkable diversity of exoproducts with antimicrobial, metal chelating, lytic, and phyto hormonal activity, and some of them have a determinative role in disease suppression and plant growth stimulation by increasing the availability and uptake of mineral nutrients (Egamberdieva et al., 2011; Glick, 2012; Kamilova et al., 2005). Indeed, diverse studies suggested that, these molecules can be bacterial signals triggering Induced Systemic Resistance (Leeman et al., 1996; Meziane et al., 2005). This is an essential prerequisite for bio control efficacy of fluorescent pseudomonads, depending on traits that allow effective root colonization and competition for nutrients and niches (CNN), that do not involve antibiotic production. It has an additional advantage that does not raise because of resistance development. Therefore, some *Pseudomonas fluorescens* can be considered to be safer and more practical than biocontrol strains which exhibit direct antagonistic activities against phytopathogens (Chen and Ljung, 2013; Cho et al., 2015; Lugtenberg et al., 2001).

The selection of highly competent pseudomonads requires expensive field sampling and fastidious laboratory analysis, thus predictive modelling using different type of mathematical models is an alternative way to describe and explain complex systems dynamics, which are known to be in practical multidimensional search problems with a number of local optima, which donot always give a satisfactory results (Weuster-Botz, 2000). Parameter optimisation techniques allow fair and objective assignment of parameter values so that, any differences in model performance can be attributed to differences in model structure, rather than to the relevance of the parameter values (Friedrichs et al., 2007). Parameter values assignment in biological modelling can strongly affect model performance, which can be hard to define accurately and precisely. In spite of that, a situation parameterisation is often used, according to the results of laboratory based studies (Pahlow and Oschlies, 2009).

The model calibration of the differential equation–based model has been applied to estimate unknown parameters and model predictions which have been quantitatively validated against experimental data and predict the time variations of substrate concentrations(iron) for which we have no experimental measurements to compare with. In addition, a sensitivity analysis in the vicinity of the resulting optimal parameter set was used to determine the strongest parameters that induce the largest changes in model's state variables.

MATERIALS AND METHODS

Bacteria strain and culture conditions

P. fluorescens Pf-10, an autochthonous strain isolated from palm date rhizosphere in south Algeria was used in this study (Benchabane, 2005; Toua et al., 2013). The strain was characterized to be resistant to rifampicine and positive for phosphate solubilisation, for the production of indole-3-acetic acid, siderophore, 1-aminocyclopropane-1-carboxylate deaminase, and 2,4 diacetylphloroglucinol (DAPG), which makes it a potential PGPR (Toua et al., 2013). The bacterial culture was maintained as 50% glycerol stocks at −20°C in King's-B medium (King et al., 1948).

Bacterial cultures were grown for 40 h at 25°C, with shaking (200 rpm) in 500 ml Erlenmeyer flasks containing 125 ml King B medium (pH 7). To remove traces of iron, glassware was cleaned with 6 M HCl and with double distilled water. For growth and pyoverdine measurements, 1.4 mL samples were taken at time zero, and then at 1 h intervals over all incubation periods.

Measurement of growth and siderophore assay

Bacterial growth was estimated turbidimetrically at 600 nm. For estimation of dry cell weight, 10 mL sample was passed through a 0.2 µm filter paper, and the residue was dried overnight to a constant weight under vacuum at 70°C. The amount of siderophore secreted into the culture medium was determined by removing bacteria by centrifugation and measuring the absorbance of the supernatant at 400 nm.

Since the siderophore produced by Pf-10 strain tested positive for hydroxamate type following the method of Snow (1954), the values of extinction coefficient $\varepsilon = 16{,}500 M^{-1}\ cm^{-1}$ and of molecular weight MW=1500Da were used. The siderophore concentration (g/L) was calculated using the expression (O.D) 400 nm ×MW/ε (Meyer and Abdallah, 1978).

Presentation of the mathematical model

The model used in the present study is derived from the well-known Baranyi's model (Baranyi and Roberts, 1994) which is widely used for its large applicability under different dynamic environmental conditions, and for the biological interpretability of its constitutive parameters (Van et al., 2005). The lag phase model was taken and consists of a non-autonomous system of four nonlinear ordinary

differential equations. The underlying assumption is that, only a fraction (α) of the whole bacterial population (N) contributes to the growth process when introduced into a new environment, while the remaining cells adapt their physiological state to the new conditions (Fgaier et al., 2008).

The bacterial population is characterized by two variables, population size (N) and its lumped physiological state, expressed in terms of a function $\alpha(t)$. The chelator pyoverdine is described by its density (P). Bacterial growth depend on iron bioavailability in the system and is represented by two forms knowing: freely dissolved denoted by S, binded by chelator molecules, denoted by Q.

Model calibration

In order to get a good fit between the approximated model solution and assimilated data, the model calibration problem is formulated as well-known nonlinear least-squares (NLS) optimization problem, Where the objective function is given as below:

$$\min_{\theta} J(\theta) = \min_{\theta} w\left(\frac{1}{n_N}\sum_{i=1}^{n_N}(\widehat{N}_i - N(t_i^N;\theta))^2\right) + (1-w)\left(\frac{1}{n_P}\sum_{i=1}^{n_P}(\hat{P}_i - N(t_i^P;\theta))^2\right) \quad (1)$$

w , is a fixed constant value equals to 0.5.

Note that, the evaluation of the objective function $J(\theta)$ requires the numerical solution of the underlying model. Thus, in order to solve the scalar least-square problem (1). sequential approach in Matlab 12 are used, by solving the optimization problem (1) through a sequential quadratic programming (SQP) technique, using a stiff solver for the underlying differential equation. A linear interpolation at specific query points is performed, in order to supply more entries for experimental data, which was found to be dense at the initial growth phase and sparse for larger time period.

To approximate realistic scenarios of microbial growth, initial guesses for the dependent state variables and model pathway parameters were provided by, generation of random parameter sets (θ) using a Latin hyper cube sampling method (LHSDESIGN Matlab function) that generated 10.000 random parameter vectors in which individual parameters were sampled independently from their respective intervals. Each simulation covered a 2-day time interval after inoculation.

From the resulting calibrated model, a measure of goodness of fit for the nonlinear regression coefficient was carried out along with a post-analysis of the calibrated models thus, adjustment level and confidence regions of the model predictions is computed, together with the scatter plots of errors for N and P.

Local sensitivity analysis

To investigate the effect of each parameter on the system behaviour, logarithmic (i.e., relative) local sensitivities, s*ij*(t) at time moments *t* was calculated, according to the standard definition of Mitrophanov et al. (2007).

$$S_{ij} = \partial Log X_i(t)/\partial Log p_j = (dX_i/X_i)/(dp_j/p_j)$$

Where*Xi*(t) is the model's *i*th variable and p*j* is the model's *j*th parameter (of the model's 8 main parameters). By definition, each of these sensitivities reflects the magnitude of the relative change in a model's output variable induced by a local (i.e., small) relative change in the vicinity of the optimal parameter set θ^*.

To obtain numeric approximations of the derivatives in Equation 2, each parameter was individually perturbed by ± (10 and 20%) of its nominal value, and the derivative was approximated using the second-order central finite difference formula.

In all analyses, local sensitivities for each of the 41 evenly spaced time was calculated which discretize the total of 2 day simulation interval into 1h subintervals (that is 0, 1h etc). To compare and rank sensitivities, absolute values were used.

RESULTS AND DISCUSSION

Parameter estimation and predictions of the mathematical model

Parameter optimisation of non-linear systems is rarely a simple task, but it is theoretically possible to objectively and fairly assign optimal to model parameters on the basis of observations, so that models can be compared in terms of structure alone, by using formal optimisation techniques (Friedrichs et al., 2007) . In the present work, the parameterisation of the governing system of differential equations is set according to real data entering results obtained from laboratory based study on bacterial growth and pyoverdine production, the integration of the system was performed for a sufficiently long time period to reach the growth limiting plateau; no stability problems have been noticed.

However, model solution as well as the values of the objective function, have been found to be sensitive to the value of the free factor (w). Reliable solutions were obtained in our simulation experiments as, a solution of the scalarized vector optimization problem θ, which was set up to be a balanced design model, and did not give advantage to biomass production termat the expense of pyoverdine content (Table 1). Conversely, with the current design, the model solution is in favour of biomass production giving rise to goodness of fit which is always superior to that of pyoverdine contents.

Experimentally measured data were successfully captured over the entire period with model fit, the quality of agreement between experimental and predicted results provide confidence in the assumptions and mechanisms in which the mathematical model is built as we correspondingly get a goodness of fit of 0.94 and 0.84 (Figure 1A and B) for biomass (N) and Pyoverdine (P). To get a visual representation of the quality of fit, model solutions along with experimental data were shown.

For pyoverdine (P), quantitative fit was also good for much data although, some discrepancies occurred from the onset stage of the exponential growth phase, even if we'veinitially performed an interpolation to supply more data points to the assimilated data.This lack of fit can be explained by the fact that observations are currently insufficient to accurately constrain the number of parameters required by even the simplest biological models, which share a common typical large variability due to randomness and underdetermination inherent to

Table 1. Initial, optimal and parameter range variation vectors associated with biomass and pyoverdine content.

Parameters symbols	Initial values	Optimal values	Range
K (µM)	1.604	2.3326	[0 - 4]
δ (µM)	0.127	0.0412	[0 – 0.13]
B (h^{-1}/OD)	0.857	0.4109	[0 – 1]
σ (h^{-1}/µM)	0.089	0.0534	[0 – 0.9]
Yn (µg $µl^{-1}$)	0.329	0.4952	[0 – 1]
µ (h^{-1})	0.122	0.1115	[0 – 0.5]
S^{∞} (µM)	0.609	0.6857	[0 – 0.7]
v	0.478	0.3831	[0 – 0.5]
State variables	**Estimated variables**		
N (OD)	0.05	0.0896	
S (µM)	$Log_{10}(100)$	10^2	
P (OD)	0.001	0.01	
Q (µM)	0	0.0617	

Legend: Yn(µg µl-1), growth yield constants, commonly referred to as the substrate-to-biomass yield factor, used to convert between cell growth rate and substrate utilization; µ (h-1), specific growth rate as a function of substrate concentration; K (µM) , is the value of the concentration of nutrients S where the specific growth rate µS has half its maximum value (half-growth concentration rate); S^{∞} (µM), iron concentration triggering pyoverdine synthesis; σ (h-1/µM), coefficient related to iron chelation rate; v(Unitless), recovery rate of PGPR population; both δ (µM) and β (h-1/OD) are coefficients related to linear functions dependent on the amount of freely available iron in the system; α(t), function of physiological adaptation state given by d fun.

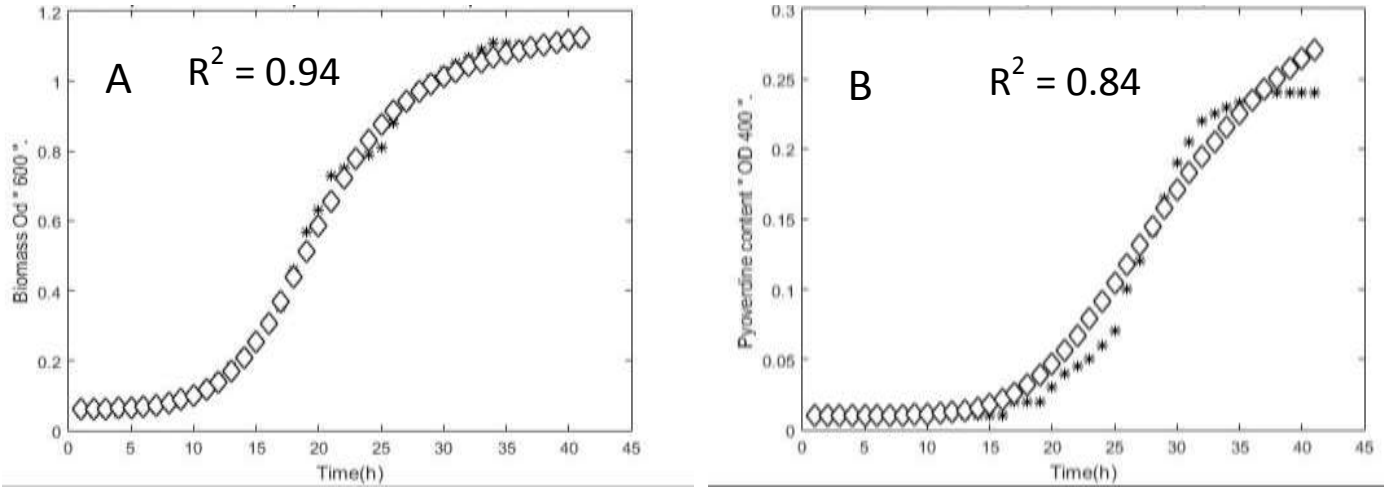

Figure 1. (A) Quality of the fit between experimental vs. predicted growth rate. (B) Quality of the fit between experimental vs. pyoverdine content.

inverse modelling problems, where the uniqueness of estimated coefficients is not guaranteed by any optimal point solution (θ^*).

In addition, the lack of fit may occur because of uncertainties related to variables that cannot be measured, missing data, physical forcing and initial conditions or plainly due to lack of knowledge (Banga and Balsa, 2008; Friedrichs et al., 2007, Kravaris et al., 2013). As a consequence, several parameters could be set anywhere across a wide range of values while providing a similarly good fit to the assimilated data (Groetsch and Groetsch, 1993). Last, this lack of fit may have been due

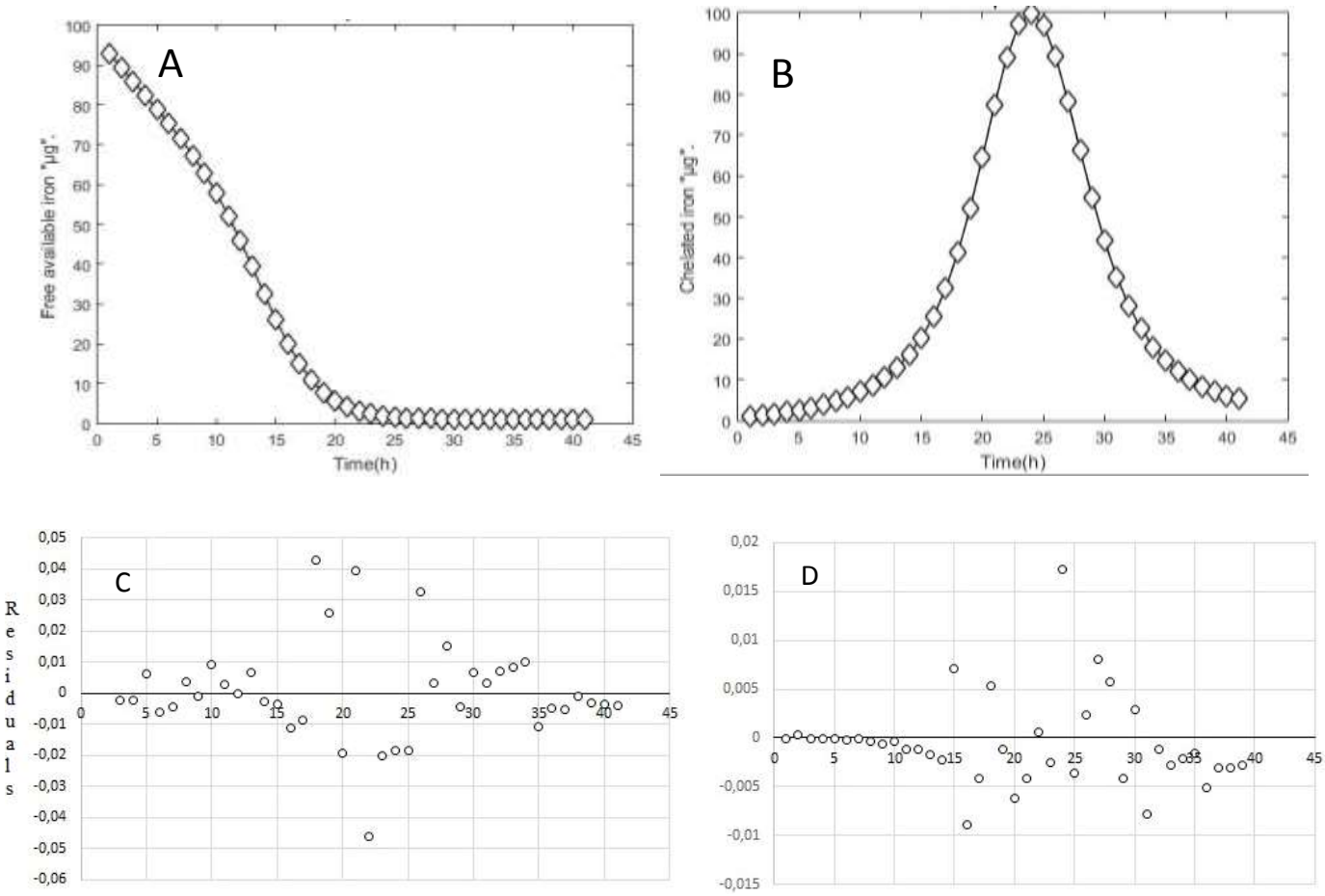

Figure 2. (A) Model prediction for free available iron (µg). (B) Model prediction for chelated iron(µg).;Residual plots for biomass production (C); and pyoverdine (D).

to experimental variability which would require more sensitive and precise experimental systems and techniques or some structural deficiencies in the underlying model assumptions, as it does not account for:

i. Substrate consumption for maintenance,
ii. Substrate breakdown in the medium,
iii. Additional substrate added during the growth process,
iv. More than one limiting substrate, and finally
v. Competition between more than one bacterial population.

In Figure 2A and B we include the corresponding predicted concentrations of freely available iron (S) and chelated iron (Q), for which we do not have measurements to compare with; together with residuals plots (Figsure 2C and D) for biomass (N), and pyoverdine content (P), which shows a mean expectation of zero and non-random small residuals values of the nonlinear growth kinetic model.

Robust sampling scheme was used such as the Latin hypercube method to helped the optimisation routine process to converge reliable solutions, by generating uniformly sampled initial guesses from each respective range of bounded domain (Table 1).This prevented each parameter from taking unrealistic values falling outside the certain range as reported by parameters mean values and solution region in Table 2, which was calculated each term of the optimal parameter vector θ^*, yielding a goodness of fit superior or equals to 0.7 over all simulations runs.

However, this approach has some disadvantages as in general; it does not allow all possible outcomes to be evaluated, such as all stationary states, periodical and chaotic regimes, or how state variables depend on the parameters (McKay et al., 1979; Schartau and Oschlies, 2003), confidence interval (CI) of the best optimal parameters vectors $\theta^{*\prime}s$.

Globally, model performance is rather good, the dimension of the solution region is consistent and the misfit costs are statistically indistinguishable from the absolute minimum, since all reaction parameters are

Table 2. Confidence interval (CI) of the best optimal parameters vectors θ^*.

Parameter	Mean value	CI (95 and 99%)
K (µM)	1.9380	[1.8320; 2.0441]
δ (µM)	0.0366	[0.0333; 0.0398]
β (h^{-1}/OD)	0.5191	[0.4999; 0.5383]
σ (h^{-1}/µM)	0.2168	[0.1974; 0.2361]
Yn (µg $µl^{-1}$)	0.5774	[0.5626; 0.5922]
µ (h^{-1})	0.3569	[0.3428; 0.3709]
S^{∞} (µM)	0.4634	[0.4484; 0.4785]
v	0.1921	[0.1821; 0.2021]

robust, that is no strong variations are found (Table 2). There is as an indication that, the model is not over-parameterized (model with large number of parameters) or over-fitted, i.e. the goodness of fit is due to the flexibility and model structure that indeed gives a robust description of the available data, without resorting to model recalibration, except for the freely available iron on which we've initially performed a $\log_{10}$ transformation in order to get consistent outputs results.

On the other hand, and contrary to many predictive models that based on experimental data, to impose a mathematical structure which pre-specifies a fixed maximum population density N_{max}, the present predictive model can cope with any maximum population density induced by essential substrate depletion, which is able to predict the amount of pyoverdine along with the freely available and chelated iron from growth measurements and vice versa, during any desired culture growth period (Gábor and Banga, 2015; Van et al., 2005) .

Model robustness analysis

Parameter robustness analysis

As the impact of model parameters on model outputs sensitivity changes over time, time-dependent parameter sensitivity analysis has been proposed to study the effect of parameter variation on model output at different time (Liu et al., 2005; Savageau, 1971). This is much more evident, since some parameter may have a positive impact on the change of a model output at some stage, and precipitously switch from positive to negative due to the complex feedbacks, associated with biological networks.

Therefore, one needs to know not only which parameters are critical for affecting model output, but also at which time do point change occur and how long it last. For this aim, the robustness of our results were tested by, performing local sensitivity analysis in the vicinities of 10,000 randomly selected parameter sets.

We identified the parameters, which induced the largest variations in the concentrations of four state variables across 10,000 simulations, ran with random parameter sets. Globally, we verified that for > 70% of the simulations, the sensitivities of the two parameters (µ) and (Yn) were the most important for majority of the considered time points simulations, with respectively 39 and 34% occurrence frequency when all state variables are considered (Figure 2). Below, representative simulation results for 2 days (8,16 and 24h…) are shown in Figure 3 aimed at each of the 10,000 randomly selected parameter sets. This is the parameter at time (8 to 16 h) after inoculation, for variable microbial biomass (N). However, this sensitivity tend to decrease over time in favor of the parameter (Yn). Conversely, for variable pyoverdine content (P), the sensitivity of the parameter (Yn) tend to increase over time to become the unique most sensitive parameter at the end of the simulation. This increase occurs during the lag-pahse (1 to 18 h after inoculation) at the expense of the specific growth rate (µ) and recovery rate (v) of *P. fluorescens* pf 10, when grown in a new media. Commonly, Pseudomonas strains have to adapt, grow and eventually produce sufficient amount of pyoverdine molecules to chelate the freely available iron present in the culture broth media to use it in its own metabolism (Swinnen et al., 2004).

On the other hand, variables related to substrate availability show that, the freely available iron (S), is most sensitive to parameter (µ). However, this parameter tend to decrease in occurrence frequency as time goes by to the advantage of (δ); (Yn) and (σ), in order of decreasing effect magnitude. For sensitivities, the later three aforementioned parameters represented less than 30% occurrence frequency while (k, Beta, S^{∞}) did not exceed 5% occurrence each. Finally, chelated iron (Q), is exclusively sensitive to the parameter (δ) at the earlier phase of bacterial growth (Lag-phase: 1to 8 h), this sensitivity tends to decrease over time and become null when the whole quantity of freely available iron is chelated and uptaken into the microbial cytosol after 24 h of microbial growth. This phase correspond to the increasing part of iron chelation curve shown in (Figure 2B).

The decrease of (δ) is associated with an increase in

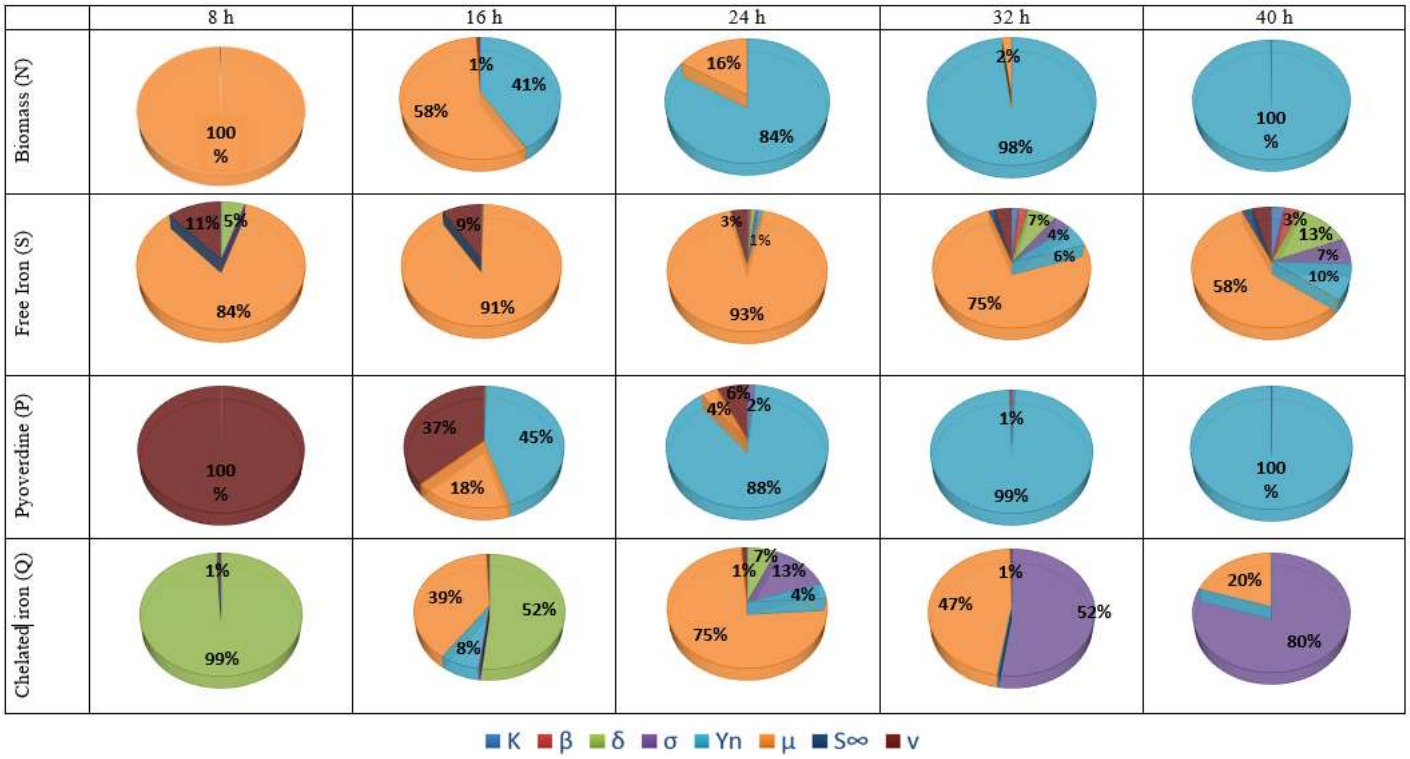

Figure 3. Summary in percentage of local sensitivity analysis results for 10,000 randomly generated parameter sets for, 5 representative discrete time points.

the sensitivity of (μ) which reach its maximum, after roughly 24 h of microbial growth then decrease under the effect of (σ) which gradually become the most sensitive parameter with higher occurrence frequency. The latter, starts from the middle stage of the exponential growth phase and last until the last stage of growth is reached (stationary phase). In parallel, this phase is associated with the decreasing phase of iron cheation curve shown in Figure 2B.

Notably, the parameters (μ) and (v) induce changes that are negatively related to the parameters (Yn); (δ) and (σ), since they all induce changes in opposite directions over time. Specifically, a decrease of (δ) is associated with an increase of (μ), and inversely, an increase of (σ) and (Yn) is associated with a decrease in the sensitivity of (μ) and (v) (Figure 3). Overall, varying model parameters in the vicinities of 10,000 randomly selected parameter sets, permited to demonstrate that the model is far more sensitive to some parameters than the others. In particular, the parameters is associated with microbial growth (μ, v); Chelate amount (Yn) and iron availability (δ, σ). However, those results can vary if one change the value of the scale parameter w in the cost function, which was set to be equal to 0.5, to avoid giving advantage for microbial growth at the expense of pyoverdine synthesis rate (balanced design), over all simulations.

Conclusion

Dynamic modelling gives an efficient structure to comprehend process in natural system frameworks. Parameter estimation in nonlinear dynamic modelling remains an extremely difficult task when inverse problems is dealt with because of their non-convexity and ill-conditioning state related to overfitting and local solutions. In modelling studies, if the long-term behaviour of the population is of interest the lag phase period can often be ignored, as it is the situation with presumably most applications in biological modelling which focus on persistence and survival.

In the simulation study, calibrated model is tested with a new data set for cross-validations by considering the same model structure and different experimental condition which adopted a computational approach focused on simulations in order to evaluate the model's validity and check agreement with experimental data. In addition, the robustness of the computational model is investigated by a local sensitivity analysis in the vicinity of the resulting optimal parameter set. Predictions of the model compared well with experimental data obtained from microbial growth and pyoverdine content measurements. In addition, they nicely predicte iron dynamic for which we did not have data to compare with.

Preceding, sensitivity analysis allowed us to pinpoint key factors (model inputs) that contribute most to the variation of model outputs.Thus, key dynamic features of the biomass and pyoverdine production can be explained and predicted using a parsimonious computational modelling approach.

However, on the other side one should be cautious since the numerical solution assumes that all parameters are independent random variables.Thus, further studies should consider the analysis of the error covariance matrix for the optimal parameters, in order to determine (i) poorly-constrained parameters (ii) correlated and partially correlated parameters (iii) how under-determination is handled by optimisation approach (vi) the exact number of parameters that could be constrained by the available data and (vii) a sensitivity analysis handling the interactions amongst more than one parameters.

Finally, the presented model approach could be useful not only as an indirect experimental tool for evaluating empirical data-based theories, but also as the basis for microbial biomass and secondary metabolite prediction which would reduce or eliminate the need for expensive laboratory analysis procedures, and help for the design and optimization of operation of large-scale production of microbial inocula and developing practical criteria in making PGPR selections, which are known to provide effective rhizosphere colonization. In perspective, further studies should focus on biofilm modelling since the interaction of pseudomonads population which are known to be natural biofilm formers and resource dynamics can be different from those occurring in suspended state media.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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