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STUDY OF DOUBLE-SUBSTRATE LIMITED GROWTH OF *PSEUDOMONAS AERUGINOSA* IN AEROBIC BIOPROCESS

Cristina TĂNASE[a], Ana Aurelia CHIRVASE[b], Camelia UNGUREANU[a*], Mihai CARAMIHAI[a] and Ovidiu MUNTEAN[a]

[a] Politehnica University of Bucharest, Department of Bioengineering and Biotechnology, 313 Splaiul Independenței, 060042, Bucharest, Roumania
[b] National Institute of Research and Development for Chemistry and Petrochemistry, 202 Splaiul Independenței, 060021 Bucharest, Roumania

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The research to put into operation an aerobic batch bioprocess for the preparation of a therapeutic immune modulator product with a strain of *Pseudomonas aeruginosa* demonstrated the important oxygen uptake during the first period of 4-6 hr of aerobic growth, which introduced the hypothesis that the dissolved oxygen can be considered as a second growth substrate. The confirmation of this finding was to be done by the growth kinetic modeling, namely by studying a double-substrate limited growth model. The interacting model that describes the growth rate as a function of both limiting substrates at the same time, the amine nitrogen and the dissolved oxygen, was considered based on the experimental behavior. The most used growth kinetic models, namely Monod, Contois and Tessier was taken into consideration to express the limitation by each of the two substrates.

INTRODUCTION

The research to put into operation an aerobic batch bioprocess for the preparation of a therapeutic immune modulator product with a strain of *Pseudomonas aeruginosa* demonstrated the product formation is growth-associated and the improving of product formation was determined by the increase of the cellular growth rate.[1]

According to the experimental data, the bacterium growth rate is big enough during a first period of the exponential phase with duration of 4-6 hr, but during a second period the cellular growth rate is characterized by an important decrease; that is why it is economically suitable to stop the cultivation bioprocess at the end of this first stage.

This behavior is in connection with the dissolved oxygen uptake, put into evidence by the pO_2 (the dissolved oxygen concentration) monitoring during the bioprocessing: the early cellular growth period is intensively aerobic, by comparison with the late period, rather microaerophilic, the microaerobic trend being characteristic for this bacterium.[2,3]

The important oxygen consumption during the start period of the aerobic growth introduces the hypothesis that it can be considered as a second growth substrate, besides the amine nitrogen, already put into evidence as growth and product formation substrate. This finding can be validated by the growth kinetic modeling, namely by studying a double-substrate limiting growth model.

There is not a large amount of dedicated information in the domain of double-substrate limiting growth kinetic modeling and less regarding the case when one of the substrates is oxygen.[4,5] Hence, the first step is to choose between the possible situations concerning the interrelationships of growth rate and essential substrates concentrations .[6-10]

According to the growth behavior characteristics several cases are described, but

* Corresponding author: ungureanucamelia@gmail.com, tel.: +40723239120

among them two situations are more frequent: (a) the non-interacting model, when the growth rate of the biological population is controlled by one limiting substrate at one time;[11] (b) the interacting model that describes the growth rate as a function of both limiting substrates at the same time, interactive nutrients being those that simultaneously affect the growth rate of the organism.[12,13] This last case is perceived when both nutrients are present in less than saturating concentrations.

In connection with the growth characteristics determined for the aerobic cultivation of the studied strain of *Pseudomonas aeruginosa* bacterium the interacting model is suitable, meaning both substrates, amine nitrogen and oxygen, are simultaneously limiting the growth rate. In this respect, there is until now only one significant study regarding this type of kinetic model for another strain of the same bacterium[14], but the cultivation conditions and the first limiting substrate are different.

Consequently, the aim of this paper is to analyze the double-substrate limiting growth kinetic models of *Pseudomonas aeruginosa* feeding on peptone under intense aerobic conditions. At the same time the kinetic models can be further used to develop bioprocess advanced and adequate global control tools[15-17] or at least to design the control of dissolved oxygen concentration.[18]

EXPERIMENTAL

Microorganism and growth conditions

A pure culture of *Pseudomonas aeruginosa* isolated from a specific hospital environment was used throughout the study. The cultivation medium was mainly a solution of 42 L aqueous Organotech® peptone and meat extract, including the inoculum of 3 L pure cellular suspension, previously grown in the same medium on a rotary incubator.

Experimental setup

The bioprocess was performed in a bottom driven 100 L Bioengineering AG pilot bioreactor in the batch mode of operation.

The bioreactor had mechanical stirring (Rushton impeller) and main parameters (temperature, pH, mixing speed, air flow rate, pO_2) were controlled, but foam level was only monitored.

The operating parameters were: temperature of 37 ^oC; pressure of 0.5 bars; mixing rate of 300 rpm; air flow rate of 40 L/min, and Dow Corning Antifoam M (added until max. 0.4 % of the medium volume), duration of 4 - 6 hours.

The cellular growth was estimated by: (a) optical density (O.D.) measurement at λ = 570 nm on a UV-VIS spectrophotometer (Jenway Spectrophotometer); (b) dried weight determination by the usual drying procedure at 105 ^oC in a thermostatic oven. The substrate consumption was measured by amine nitrogen consuming determination (chemical titration with NaOH in formaldehyde presence).[1]

The data modeling was done in the program Matlab R 2010 a.

RESULTS AND DISCUSSION

Choice of the growth kinetic models

In the general case of the interacting model of two essential substrates the kinetics, that expresses the specific growth rate function of double substrate limitation[6], is:

$$\mu(C_{S1}, C_{S2}) = \mu_{max} r(C_{S1}).r(C_{S2}) \qquad (1)$$

where:

C_{S1}, C_{S2} – concentrations of the limiting substrate 1 and respectively 2 [mg/L]

μ - microbial specific growth rate, [h^{-1}]; μ_{max} – the maximum specific growth rate

r – normalized kinetics without inhibition

The normalized kinetic expressions for each substrate limitation, in this specific case limitation by both, the amine nitrogen and the dissolved oxygen, can be chosen among the most applied kinetic models $\mu=\mu(C_S)$ or $\mu=\mu(C_S, C_X)$. It is finally to consider above all the models Monod, Tessier and Contois, and to propose the one another combinations conforming to the relationships presented in the Table 1.

The experimental data sets from 4 batch bioprocesses were used to check the adequacy of the proposed models, and the most representative results are outlined further on.

The experimental data from these batch bioprocesses are presented in Fig. 1.

The representations of the estimated curves by the equations (2) - (10) for the selected 4 batch bioprocesses by comparison with the experimental data evolutions were done, demonstrating a good enough fitting. For instance a representation of the experimental data versus the curves obtained with the model type B, equation (3), is done for batch 4 in Fig. 2.

Further on the best double-substrate limiting growth models were selected based on the criterion of the minimum sum of squares of differences (SSD) between the experimental data and the model solutions determined by applying the above mentioned equations. The interacting model was proved adequate for five combinations of simple growth kinetic models, namely Tessier-Tessier, Contois-Tessier, Contois-Monod, Tessier-Monod, and Monod-Tessier.

Table 1

Representative kinetic models

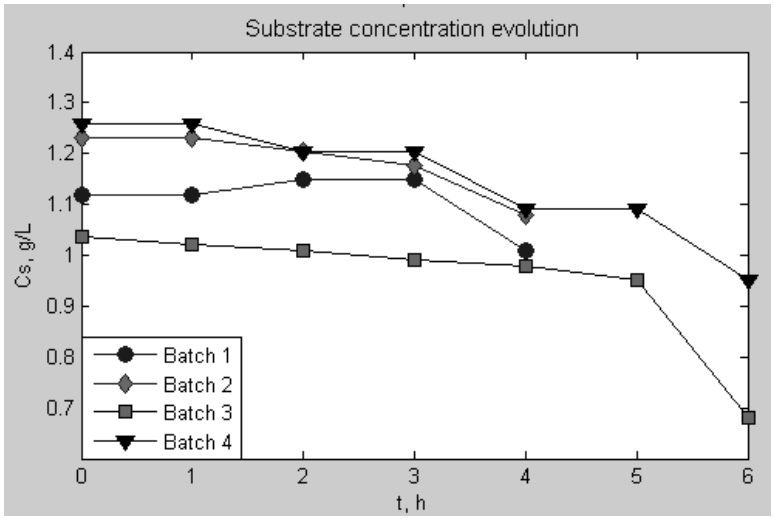
Model Type	Representative equation
A. Monod-Monod (Olsson) (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O + C_O} \cdot \frac{C_S}{K_S + C_S}$ (2)
B. Tessier-Tessier (oxygen-amine nitrogen)	$\mu = \mu_{max} \left(1 - e^{-\frac{C_O}{K_O}}\right) \cdot \left(1 - e^{-\frac{C_S}{K_S}}\right)$ (3)
C. Contois-Monod (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O \cdot C_x + C_O} \cdot \frac{C_S}{K_S + C_S}$ (4)
D. Contois-Tessier (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O \cdot C_x + C_O} \cdot \left(1 - e^{-\frac{C_S}{K_S}}\right)$ (5)
E. Monod - Tessier (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O + C_O} \cdot \left(1 - e^{-\frac{C_S}{K_S}}\right)$ (6)
F. Tessier- Monod (oxygen-amine nitrogen)	$\mu = \mu_{max} \left(1 - e^{-\frac{C_O}{K_O}}\right) \cdot \frac{C_S}{K_S + C_S}$ (7)
G. Monod - Contois (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O + C_O} \cdot \frac{C_S}{K_S \cdot C_x + C_S}$ (8)
H. Tessier - Contois (oxygen-amine nitrogen)	$\mu = \mu_{max} \left(1 - e^{-\frac{C_O}{K_O}}\right) \cdot \frac{C_S}{K_S \cdot C_x + C_S}$ (9)
I. Contois - Contois (oxygen-amine nitrogen)	$\mu = \mu_{max} \cdot \frac{C_O}{K_O \cdot C_x + C_O} \cdot \frac{C_S}{K_S \cdot C_x + C_S}$ (10)

where:
C_s – amine nitrogen concentration [mg/L]
C_o – oxygen concentration [mg/L]
C_x – cell's concentration [mg/L]
K_o – half-saturation constant for oxygen [mg/L]
K_S – half saturation constant for amine nitrogen [mg/L]

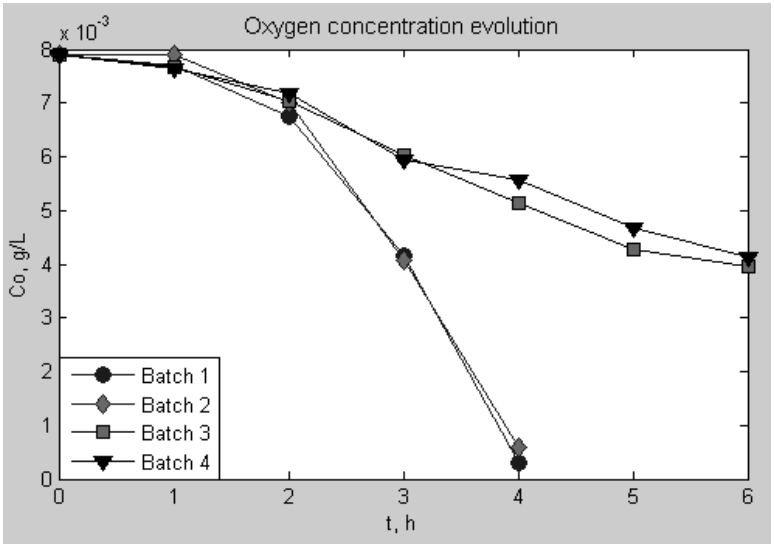
The synthesis regarding the calculated models' parameters (for these best double-substrate limiting growth models) in all the 4 batch bioprocesses is presented in the Table 2.

To further check the validity of the proposed kinetic models with double substrate limitation it was necessary to compare the maximum specific growth rate values determined by applying the kinetic models and represented in the Table 2 with the values calculated based on the experimental data. The maximum specific growth rates determination considering the experimental data was done by using the exponential model.[1]

Finally the correlations between the experimental maximum specific growth rates and the maximum specific growth rates obtained by applying the proposed kinetic models were determined. Table 3 shows the values of the regression coefficients estimated for the selected bioprocesses in case of each interacting kinetic model with double substrate limitation.



(a)



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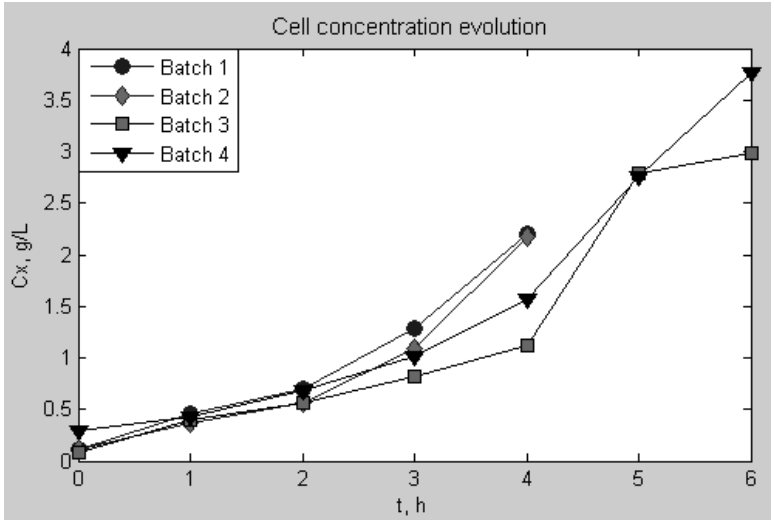
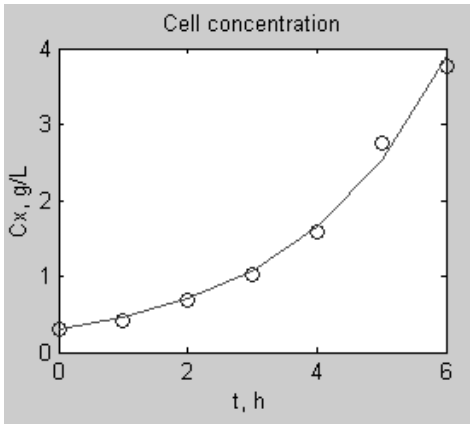


(c)

Fig. 1 – The experimental data from the batches 1-4 used in the kinetic modeling:
(a) substrate concentration evolution; (b) pO_2 evolution; (c) cells' concentration evolution.



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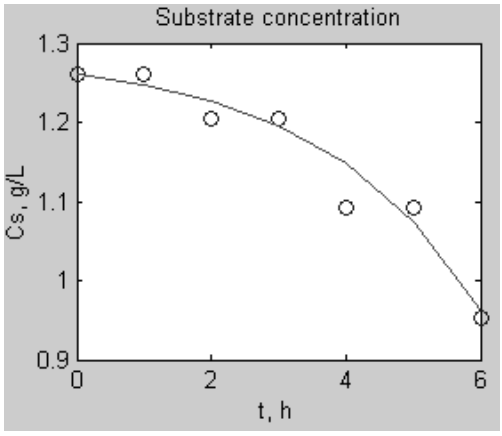

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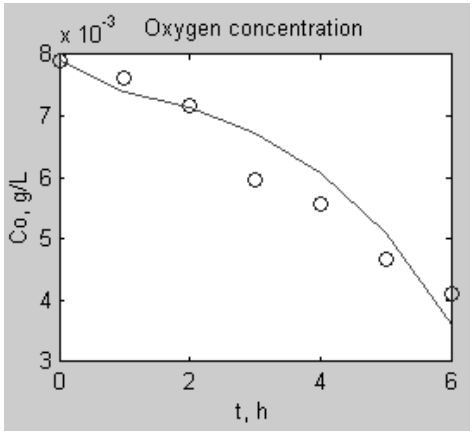

(c)

Fig. 2 – The cellular concentration evolution (a), the substrate (amine N) evolution (b), and the O_2 concentration evolution (c) versus time, batch 4 (in points-real data; green curve-simulation with the equation (3)).

Table 2

Parameters of the kinetic models, in case of 4 representative batch bioprocesses

Batch	Model Type	μ_{max}, h^{-1}	K_O, g/L	K_S, g/L	SSD	R^2
1	**Tessier-Tessier**	0.8475	0.0011	0.0032	0.1937	0.9988
	Contois-Tessier	0.9851	0.0005	0.0021	0.1650	0.9665
	Contois-Monod	0.8696	0.0002	0.0012	0.0903	0.9988
	Tessier-Monod	0.8435	0.0011	0.0032	0.2223	0.9991
	Monod-Tessier	0.9986	0.0008	0.0027	0.0571	0.9988
2	**Tessier-Tessier**	0.7966	0.0009	0.0027	0.0060	0.9988
	Contois-Tessier	0.8114	0.0002	0.0032	0.0044	0.9665
	Contois-Monod	0.8129	0.0002	0.0023	0.0044	0.9988
	Tessier-Monod	0.7901	0.0008	0.0026	0.0131	0.9991
	Monod-Tessier	0.8729	0.0005	0.0025	0.0042	0.9988

Table 2 (continued)

3	**Tessier-Tessier**	0.7005	0.0010	0.0035	0.0892	0.9988
	Contois-Tessier	0.7036	0.0001	0.0039	0.0868	0.9665
	Contois-Monod	0.7049	0.0001	0.0020	0.0868	0.9988
	Tessier-Monod	0.7050	0.0011	0.0036	0.0898	0.9991
	Monod-Tessier	0.7387	0.0004	0.0052	0.0865	0.9988
4	**Tessier-Tessier**	0.4307	0.0003	0.0052	0.0410	0.9988
	Contois-Tessier	0.4165	0.0001	0.0066	0.0036	0.9665
	Contois-Monod	0.4186	0.0001	0.0062	0.0036	0.9988
	Tessier-Monod	0.4358	0.0013	0.0035	0.0636	0.9991
	Monod-Tessier	0.4764	0.0007	0.0018	0.0797	0.9988

Table 3

The regression coefficients calculated for the experimental μ_{max} versus μ_{max} done for each of the five interacting kinetic models and considering four bioprocesses

Model Type	**R^2**
Tessier-Tessier	0.9988
Contois-Tessier	0.9665
Contois-Monod	0.9988
Tessier-Monod	0.9991
Monod-Tessier	0.9771

The values of the maximum specific growth rate determined by the proposed models are high, in the domain of 0.4 to 0.9 h^{-1}, for the 4 batch bioprocesses, but in good agreement with the experimental levels. At the same time these values have the same order of magnitude, but are higher when compared with the data presented in the literature (0.3 to 0.45 h^{-1}) for double substrate kinetics also modeled for a *Pseudomonas aeruginosa*[14] bacterium, but in case of another strain, grown on a medium with glucose as substrate in chemostat.

The values of the regression coefficient are also high, more than 0.9 for the five proposed kinetic models with double substrate limitation; this finding validates the bacterium growth can be modeled by double substrate limitation kinetics and shows that both the amine nitrogen and the dissolved oxygen can be considered as interacting limiting substrates. The models with the highest adequacy were Tessier-Monod, Tessier-Tessier and Contois-Monod ($R^2 >$ 0.99), followed by the models Monod-Tessier and Contois-Tessier ($R^2 < 0.98$).

In case of the double Tessier kinetics (Eq. 3) the estimated values of K_O - half-saturation constant for oxygen varies from 0.3 to 1.1 mg/L (Table 2), and are similar to the value of 1.18 mg/L determined in the already described conditions for another strain of *Pseudomonas aeruginosa*; so one can consider that this is a typical metabolic characteristic of the bacterium growth. There are no data to compare K_S levels, because one cannot find until now in the dedicated literature other cases where the limiting substrate for the studied bacterium is amine nitrogen.

CONCLUSIONS

The research to put into operation an aerobic batch bioprocess for the preparation of a therapeutic immune modulator product with a

strain of *Pseudomonas aeruginosa* introduced the hypothesis that the dissolved oxygen can be a second growth limiting substrate, besides the amine nitrogen, already considered as growth and product formation substrate.

The interacting model that describes the growth rate as a function of both limiting substrates at the same time was proposed based on the experimental behavior.

The simple growth kinetic models Monod, Contois and Tessier were selected to express each substrate limitation of the specific growth rate.

This interacting kinetic models with double substrate limitation was proved adequate for five combinations of the chosen models, namely Tessier-Tessier, Contois-Tessier, Contois-Monod, Tessier-Monod, and Monod-Tessier, based on the determination of the minimum sum of squares of differences (SSD) between the experimental data from several batch bioprocesses and the model solutions.

The correlations between the experimental maximum specific growth rates determined by using the exponential model and the maximum specific growth rates predicted by each of the chosen interacting kinetic models allowed choosing the cases characterized by the best regression coefficients (R^2). The models with the highest adequacy were Tessier-Monod, Tessier-Tessier and Contois-Monod ($R^2 > 0.99$), followed by the models Monod-Tessier and Contois-Tessier ($R^2 < 0.98$).

The modeling study validated the bacterium growth can be modeled by double substrate limitation kinetics, and demonstrated both the amine nitrogen and the dissolved oxygen can be considered as interacting limiting substrates.

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