VARIATIONS AND MODELLING OF OXYGEN DEMAND IN AMINO ACID PRODUCTION

H. M. Yao, Y. C. Tian*, M. O. Tadé and H. M. Ang
School of Chemical Engineering, Curtin University of Technology
GPO Box U1987, Perth WA 6845, Australia
Fax: +61-8-9266-3554
E-mail: {yaoh, tiany, tadem, angm}@che.curtin.edu.au

ABSTRACT

Keywords: L-lysine, fermentation, dissolved oxygen tension (DOT), modeling, optimization

1. INTRODUCTION

Amino acid is the building blocks for protein, which are required for healthy growth and development in man and in many domestic animals. When diets are lacking in one or more essential amino acids, this may give rise to characteristic deficiency diseases. Lysine is one of the twenty-two common amino acids found in protein, and it is not biologically synthesized by the body itself or at least not sufficient to meet the body's needs for normal growth or maintenance. Its content in grains except soybean, oilseed meal and other feed materials is also very low. To overcome these deficiencies, large amounts of lysine have been used as nutritional additives for supplementation of grain proteins or for improvement of growth for the animal fed.

Large-scale production of animals has attracted industrial synthesis of L-lysine. Within the two main streams of methods to produce L-lysine: direct fermentation and chemical synthesis, the use of fermentation technology accounts for 80% of worldwide production. There exists in all microbial cells a pool or reservoir of amino acids which is continually being utilized in protein synthesis. However, free amino acids may be excreted into medium, particularly towards the end of the exponential phase of growth when protein synthesis is on the decline. As all amino acid-accumulating microorganisms which are currently known are aerobes, it suggests that the accumulation of amino acids depends on a metabolic balance which may be affected by environmental factors. Many of the selected strains may undergo great changes in metabolic flow in response to changes in environmental conditions, within which Dissolved oxygen tension (DOT) is one of the important technological parameters influencing fermentation processes, the state of microbial culture and therefore the biosynthesis of the desired product. The greater availability of dissolved oxygen stimulates the further uptake of glucose and utilization of phosphate. Reduced oxygen availability could result in excretion of lactic acid and acetic acid. Under this condition, some metabolites may also act as inhibitors, e.g. sugar phosphate (glucose-6-phosphate or fructose-6-phosphate) arising from the phosphotransferase system.

In order to optimize cell growth and product formation, it is often desirable to control the DOT at a certain level (Radjai *et al*., 1984). It has also noted that different levels of DOT are favored during the different fermentation stages (Yao, Tian *et al*., 2000). For example, in order to get higher amino acid production, a lower oxygen supply during the exponential phase was favored and a high oxygen supply rate was necessary during the production phase (kwong *et al*., 1991). Thus, the design of appropriate DOT control profiles is crucial for L-lysine fermentation as well as for other amino acids production. However, only limited information is available from the open literature regarding the DOT profile study. Some initial experimental results on DOT control profiles for L-lysine fermentation has been reported recently by the authors (Yao, Tian *et al*., 2000). Experimental investigation is vital for this subject due to its costly and time consuming. To avoid these experimental difficulties, simulation studies can be carried out prior to the bench top investigation.

To investigate DOT control profiles via computer simulation, good process models are required for both growth and DOT dynamics, and the models have to be lined with the manipulated variable of the fermentation process. Only few attempts have been made to model the kinetics of lysine synthesis (Chao *et al*., 1992; Kazakov and Ganipolskiy, 1994) and the agitation/aeration-DOT channel. The qualitative control of DOT is quite complicated because the dynamic characteristic of this channel depends on the

culture state and it will change in a wide range during the whole process. Lacking of precise mathematical models describing the cell growth metabolic production limits the application of traditional model-based control theory. Our efforts have been made to set up an integrated process model that relates the DOT to the kinetic parameters with agitation speed as the manipulated variable. Because of the complicity of the fermentation process and the unusual response of DOT, this model can be used as a good example for developing advanced control of non-linear systems.

This work has been conducted in two steps. The process model was first developed based on material balances, and the kinetic parameters are identified under four different DOT conditions that ranged from 2% to 20%. These Dots selection were considered to have met different requirement for oxygen supply, e.g. from oxygen limiting (2%) to completely satisfied (20%). As for bacteria culture, the critical dissolved oxygen concentration is about 5% to 10% of the saturated value (Riviere, 1977). Next, all the kinetic parameters were expressed in the functions of DOT, the considered operating condition. Two kinds of models are presented with different prediction abilities. The selection of rate equations for the model 1 was from classical study on typical fermentation process. The second model adopted an empirical equation for lysine synthesis. With one more parameters, the overall improvement on the model prediction is significant. Both of the models are compared and discussed. The proper selection of models for different control purpose is recommended.

2. MATERIALS AND METHODS

A mutant strain of *Brevibacterium lactofermentum* (B/L M76, AEC^r, ser^-, FP^s) was employed throughout this study. The rich media contained 10.0g/L D-glucose, 10.0g/L yeast extract, 10.0g/L peptone and 2.5g/L NaCl. The composition of inoculum media contained 1.0g/L $CaCl_2 \cdot 2H_2O$, 30.0g/L $(NH_4)_2SO_4$, 0.4g/L $MgSO_4 \cdot 7H_2O$, 0.05g/L NaCl, 0.0076g/L $MnSO_4 \cdot H_2O$, 0.01g/L $FeSO_4 \cdot 7H_2O$, 0.5g/L KH_2PO_4, 0.5g/L K_2HPO_4, 2.0g/L urea, 1.0g/L yeast extract, 1.0g/L peptone, 10.0g/L D-glucose, 0.2mg/L thiamine $\cdot$ HCL, and 0.5mg/L D-biotin. The fermentation media are almost the same as IM except: 20.0g/L $(NH_4)_2SO_4$, 0.5g/L KH_2PO_4, 0.5g/L K_2HPO_4, 150g/L D-glucose and 0.1mg/L L-serine. In order to maintain a proper pH value (7.0), the concentration of $(NH_4)_2SO_4$ in inoculum media is higher than that in fermentation media where the pH is controlled by the addition of alkali.

Culture conditions for the 5L B. Braun Biostat fermentor were as follows. Two loops of cells, grown on a nutrient agar strain maintenance plate for 24h at 30°C, were inoculated into 20mL of rich media in a 250mL conical flask. After 24 hours cultivation on an orbital shaker, at 120rpm, at 30°C, the seed broth was transferred to 180mL of inoculum media in a 2L Erlenmeyer flask and put on an orbital shaker, at 120rpm, and at 30°C for 40 hours. This was then used to inoculate the fermentation media in the fermentor.

Batch fermentation experiments were carried out with 2.2L total volume for 92h. It was performed at 30°C with aeration at $1.0 L min^{-1}$. The pH was automatically controlled at 7.0 with 8.5M NH_4OH solution. The dissolved oxygen level was maintained at 10% air saturation by the cascade control of stirrer speed.

3.PROCESS MODEL DEVELOPMENT

A suitable approach based on laboratory experimental data is the development of a 'balanced' mathematical model of the system under study. This technique is based on combined use of an assumed kinetic model and a material balance for a single chemical constituent. When the material balance model is used with product and biomass, and considering substrate utilized for product formation, the following relationship can be established for L-lysine fermentation on batch mode:

Cells:

$$dX/dt = \mu X \quad (1)$$

Carbon substrate:

$$dS/dt = -r_s \quad (2)$$

Product:

$$dP/dt = r_p \quad (3)$$

It is assumed that a constant fermenter volume for the batch fermentation was maintained and the addition of alkali for pH adjustment can be neglected.

3.1 Determination of Rate Equations

The reaction rates are usually approximated by using one of the relationships derived from the theory of enzymic or chemical reaction [12]. From the results of batch fermentation (with approximately 150g/L initial glucose concentration) shown in Figure 1, the variation of the specific growth rate μ with glucose concentration in this L-lysine fermentation process could not be properly represented by the traditional Monod equation. After carefully examining the different types of reaction rates recommended by reference [12], a non-linear relationship as shown in Equation (4) was selected for this study.

$$\mu = k\, S^n \tag{4}$$

A regression analysis on the experimental data showed that the above model represents the curve in Figure 1 adequately. Further detail can be found in the reference [13]. This idea was also supported by Ohno [14] for an L-lysine production process by an auxotrophic mutant, where a first order empirical kinetic equation describing the linear relationship between μ and S was suggested.

The significance of k and n may be discussed as follows. The reaction rate following this type will increase with increase of k and n. Parameter k represents the velocity with which the substrate is utilized for the growth and n dominates the change of the rate. In the situation of the specific growth rate, a low n indicates a more balanced growth that means the exponential phase is relatively longer. In other words, a high n indicates the cell growth is more affected by the conditions. A big k will generally represent a high specific growth rate with the same concentration of substrate. Obviously, improving k and n will be beneficial to the growth. However, for the fermentation of some product, the specific growth rate is required to be maintained at a low level to stimulate a high product formation activity.

Although Luedeking - Piret model, has been popularly accepted to describe growth associated, non-growth associated or mixed product formation, the kinetics of some product formation are imperfectly understood. Another model as given in Equation (5) was selected to represent the L-lysine formation rate according to the experimental result shown in Figure 2.

$$r_p = a\, X\, Ln(\, bS + c\,) \tag{5}$$

It can be seen that **a**, **b** and **c** all have positive effect on the specific product formation rate. **a** is directly proportional to the substrate consumption and the effect will be felt through the whole process consistently. The parameter **b** may determine the peak of the rate, which is the highest specific production rate that could be obtained. The parameter **c** is interesting to investigate. In the situation of a low **c**, the product formation could stop even before the substrate was exhausted. This indicates some sort of inhibition from other than the finishing of the main carbon source.

Three other parameters for the substrate consumption need to be identified. The carbon substrate is used for three purposes: the synthesis of cells, the synthesis of complex biochemical and the energy stored as ATP used for the above synthesis and maintenance requirements.

$$r_s = \frac{r_x}{Y_{x/s}} + \frac{r_p}{Y_{p/s}} + m_s X \tag{6}$$

3.2 Parameter Identification

With the off-line measurement of biomass, glucose and L-lysine concentration, parameter identification can thus be done by Nelder and Mead optimization combined with Fourth Runga-Kuta method using Matlab software package. No secondary variables (e.g. μ, r_s or r_p) were used for the identification. The objective function was defined as:

$$J_{\min} = \sqrt{(X_m - \hat{X})^2} + \sqrt{(P_m - \hat{P})^2} + \sqrt{(S_m - \hat{S})^2} \tag{7}$$

The subscript $_m$ stands for measurement and ^ represents the model prediction.

In Figure 3, the indicated points are the measured values while the curves are predicted values. It is obvious from this graph that the model prediction adequately represents the real process.

3.3 Correlation of DOT to the Kinetic Parameters

In order to find out the influence of dissolved oxygen tension on microbial growth and product rate, parameter identification was applied to the four series of data mentioned in Section3.2. The result of the parameters related to the rate equations and some of the criteria for the model validation test are listed in Table 1. Efforts have been made to find out the relationship between those parameters and DOT. The corresponding curve fittings are shown in Figures 4. The function correlated DOT to the parameter was found via Least-square method using Matlab. Statistical analysis of the fitting results is listed in Table 3.

The functions are summarized below:

$$k = 10^{-7} \times 1.2986e^{0.47485DOT} \tag{8}$$

$$n = -(9.215e-5)DOT^3 - (9.335e-4)DOT^2 - 0.009872DOT + 2.084 \tag{9}$$

$$a = -(6.019e-7)DOT^3 + (7.731e-6)DOT^2 + (2.694e-4)DOT + 0.03834 \tag{10}$$

$$b = -(5.796e-5)DOT^2 + 0.01990DOT - 0.1965 + \frac{1.243}{DOT} \tag{11}$$

$$c = 36.6987e^{-0.15065DOT} + 0.168325DOT - \frac{84.52}{DOT} \tag{12}$$

$$Y_{x/s} = -(2.623e-5)DOT^3 + (2.307e-3)DOT^2 - 0.05179DOT + 0.5546 \tag{13}$$

$$Y_{p/s} = -(8.344e-5)DOT^3 + (3.011e-3)DOT^2 - 0.01182DOT + 0.3542 \tag{14}$$

Generally, all those kinetic parameters were affected by the dissolved oxygen tension. As mentioned before, the process with a low n has low and stable specific growth rate. This happened with 20% DOT, where n was 0.776 compared with 2.06 at 2% DOT. The advantage of slow growth is that more carbon source will be directed to the product formation. However, this process has the highest k, which maintain a certain rate of growth to accumulate enough number of cells. 20% DOT also has the highest specific product formation rate when the substrate is depleted, also it indicates sufficient oxygen supply can keep a high product formation activity. For 2% DOT, γ is negative (-14.7). In fact, this process has about 30g/L residual glucose concentration when both growth and production stopped. Because it did not observed at higher DOT, it may be concluded that insufficient oxygen supply has inhibited the complete consumption of carbon source. However, as the glucose concentration still declined without the increase of biomass and lysine, other product tended to be produced, most likely the lactic acid. 20% DOT also has higher yield of L-lysine and lower yield of biomass.

4. Modification of L-lysine Formation Rate

$$r_p = aX(1 - e^{-bS}) + \frac{X}{S + 0.001} e^{cS-d} \tag{15}$$

$$k = 10^{-5} \times 0.1217e^{0.3687DOT} \tag{16}$$

$$n = \frac{2.008}{DOT} - 0.0643DOT + 1.9449 \tag{17}$$

$$a = 12.5095e^{-1.0744DOT} - 0.0068e^{-0.1315DOT} + 0.0073DOT \tag{18}$$

$$b = \frac{4.6713}{DOT^3} - \frac{5.3532}{DOT^2} + \frac{1.5783}{DOT} - 0.03403 \tag{19}$$

$$c = 0.04342 + 0.01025DOT - 0.0004795DOT^2 \tag{20}$$

$$d = 11.3229e^{-0.0281DOT} + 0.1646DOT - \frac{5.5999}{DOT} \tag{21}$$

$$Y_{p/s} = \frac{0.1733}{1 + e^{-2.6359DOT + 22.7846}} - 0.007\sqrt{DOT} + 0.3297 \tag{22}$$

$$Y_{x/s} = 0.1132e^{0.054DOT} + 1.352e^{-0.3196DOT} \quad (23)$$

The parameter identification results are listed in Table 2 and the comparison of these two models is shown in Table 3. The corresponding curve fittings are shown in Figures 5.

5. Discussion

Generally, it can be seen that the change of dissolved oxygen tension leads to a change of the bacteria's behavior, both in growth and product formation. This is indicated by the shift away from the original trajectory of time profile. Particularly, for the oxygen content in exhaust gas, where a sudden increment was observed irrespective of whether the dissolved oxygen concentration was increased or decreased. It implies that the oxygen uptake rate has a very close relationship with the dissolved oxygen concentration. During the first 48 hours, L-lysine concentration could reach the same value as that under 20% DOT. In the later phase, 5% DOT gave a better result than sufficient oxygen supply. It may be concluded that L-lysine fermentation would prefer a slightly limited oxygen condition. The first 24 hours low oxygen supply was obviously good to the growth. The following 10% DOT was appropriate to control the growth at a limited speed however also avoid the inhibition caused by low DOT level which presented under 2% DOT.

Although the model derived from this study contains a relatively large number of equation, it will be expected to be used for the control purpose in conjunction with the model of the dynamic behavior for DOT[16]. Majority of fermentation processes requires local regulation for control. However, the varying dynamics experiences over the course of the fermentation can cause some difficulties. This challenges the controller-tuning problem. Auto-tuning controllers of more advanced model-based controllers are preferred in fulfilling the bioprocess control tasks.

REFERENCES

1. Sassi, A. Hadj; Deschamps, A. M.; Lebeault, J. M., (1996), "Process analysis of L-lysine fermentation with Corynebacterium glutamicum under different oxygen and carbon dioxide supplies and redox potentials", *Process Biochemistry*, **31(5)**, pp493-497.
2. Radjai, M. K., Hatch, R. T., and Cadman, T. W., (1984), "Optimization of Amino Acid Production by Automatic Self-Tuning Digital Control of Redox Potential", *Biotechnology and Bioengineering Symp*., **14**, pp657-679.
3. Kwong, S. C. and Rao, G., (1991), "Utility of Culture Redox Potential for Identifying Metabolic State Changes in Amino Acid Fermentation", *Biotechnology and Bioengineering*, **38(9)**, pp1034-1040.
4. Toma, M. K., Ruklisha, M. P., Vanags, J. J., Zeltina, M. O., Leite, M. P., Galinina, N. I., Viesturs, U. E. and Tengerdy, R. P., (1991), "Inhibition of Microbial Growth and Metabolism by Excess Turbulence", *Biotechnology and Bioengineering*, **38**, pp552-556.
5. Chao, S. B., Scharer, J. M. and Moo-Young, M., (1992), "A General Method for Biological Parameter Estimation", *Biotechnology Techniques*, **6**(6), pp495-500.
6. Kazakov, A. V. and Ganipolskiy, S. L., (1994), "Suboptimal Control of Batch Fermentation Processes Based on Their Classification by the Peculiarity Index", *Biotechnology Progress*, **10**, pp606-610.
7. Shuler, M. L. and Kargi, F., (1992), *Bioprocess Engineering: basic concepts*, Prentice-Hall Inc. New Jersey.
8. Stanury, P. F., Whitaker, A. and Hall, S. J., (1995), *Principles of Fermentation Technology*, 2nd Edition. Pergamon, Oxford, UK.
9. Omstead, D. R., (1987), *Computer Control of Fermentation Processes*, CRC press Inc., Boca Raton, Florida.
10. Satiawihardja, B., (1990), *Kinetics of L-lysine Production by a Fluoropyruvate Sensitive Strain of* Brevibacterium Lactofermentum, Ph.D. Thesis, University of New South Wales, Australia.
11. Hirose, T., Nakano, T., Azuma, T., Sugimoto, M. and Nakanishi, T., (1989), "L-lysine Production in Continuous Culture of an l-lysine Huperproducing Mutant of C. glutamincum", *Applied Microbiology and Biotechnology*, **32**, pp269-273.
12. Volesky, B. and Votruba, J., (1992) *Modeling and Optimization of Fermentation Processes*. Elsevier, Amsterdam.
13. Yao, H. M., (1999*) Control of Dissolved Oxygen Concentration and Substrate Feed for a Fermentation Process*. Master Thesis, Curtin University of Technology, Australia.
14. Ohno, H., (1978) *Biotechnology and Bioengineering*. **20**, p625.
15. Press, W. H., Flannery, B. P., Tuekolsky, S.A. and Vetterling, W. T., (1992) *Numerical Recipe*. Cambridge University Press, England.
16. Yao, H. M., Tian, Y. C. and Tade, M. O., (2000),"An Integrated L-Lysine Fermentation Model with Agitation Speed as the Manipulated Variable",----------

NOMENCLATURE

X: Biomass concentration in the liquid medium	g /L
S: Carbon substrate concentration in the liquid medium	g /L

P: Product concentration in the liquid medium		g /L
μ: The specific growth rate of viable cells		/h
r_s: Rate of carbon substrate consumption		g /L/h
r_p: Rate of product formation		g /L/h
$Y_{p/s}$	Yield coefficient for product on carbon substrate	g /g
$Y_{x/s}$	Yield coefficient for cells on carbon substrate	g /g
m_s	Maintenance coefficient for cells on carbon substrate	g /g/ h
a,b,c,d	Product formation coefficient	
n	Coefficient for specific growth rate	
k	Coefficient for specific growth rate	kg^{-1} S m^{-3} h^{-1}

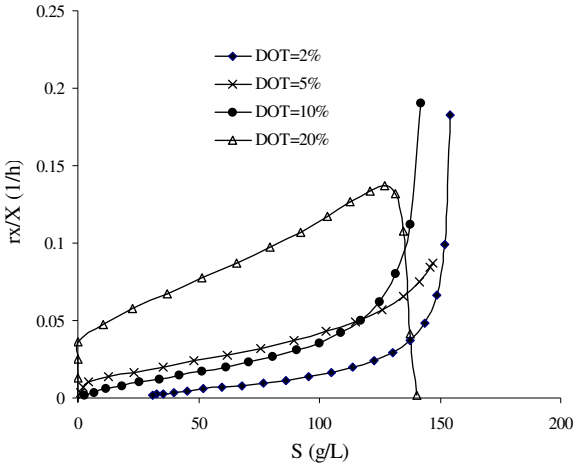


Figure 1 The relationship of specific growth rate with glucose concentration.

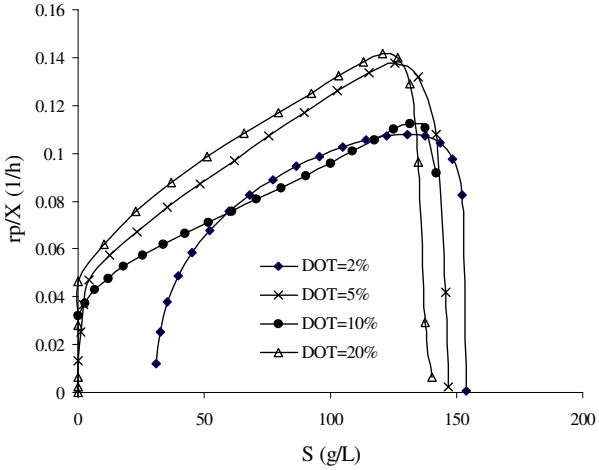


Figure 2 The relationship of the specific production rate with glucose.

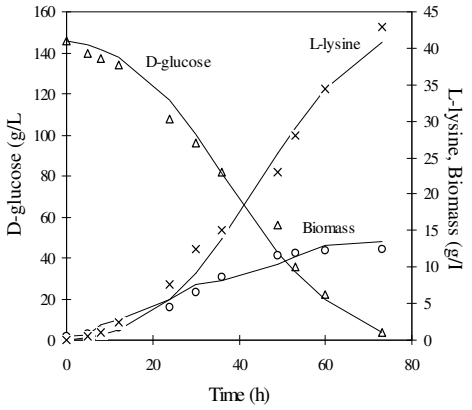


Figure 3 Model validation.

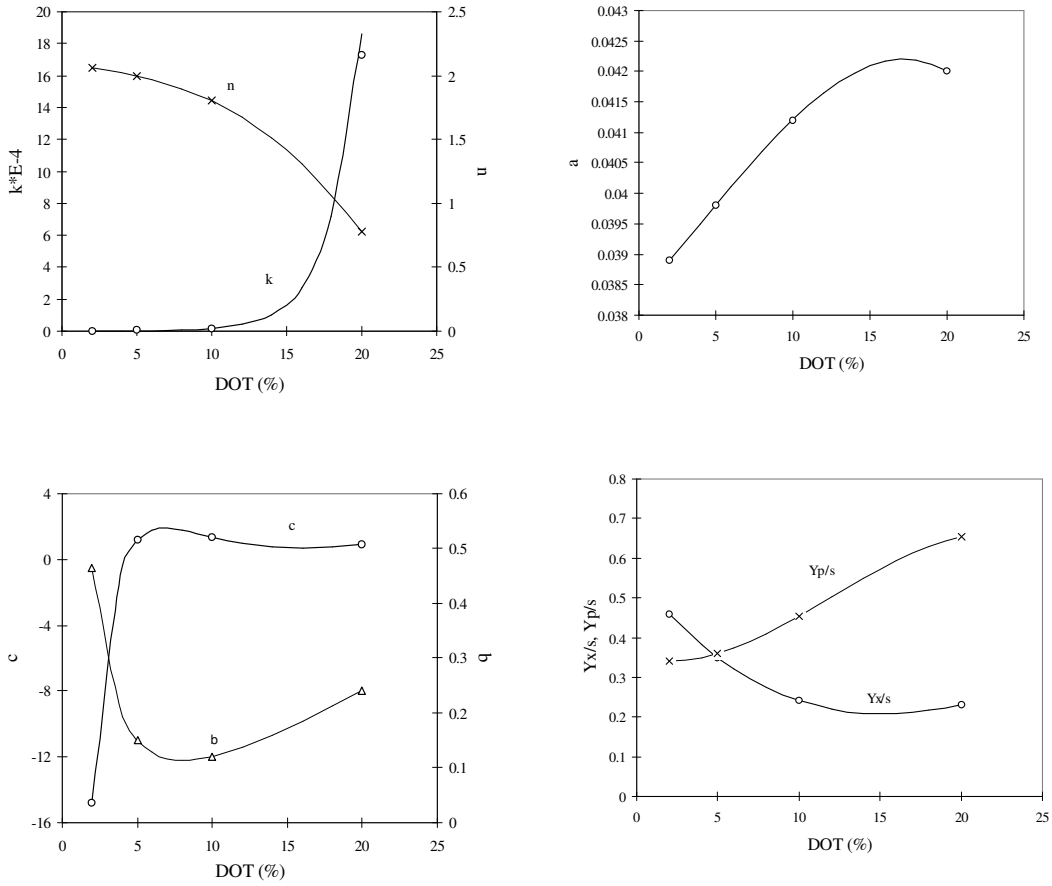


Figure 4. Correlation of DOT [2 20] to the kinetic parameters of Model 1.

Table 1. Influence of DOT on the kinetic parameters of Model 1.

DOT	k	n	a	b	c	$Y_{x/s}$	$Y_{p/s}$
20%	1.73e-03	0.776	0.0420	0.241	0.945	0.232	0.654

k

10%	1.33e-05	1.81	0.0412	0.121	1.369	0.241	0.454
5%	5.17e-06	1.99	0.0398	0.150	1.219	0.351	0.359
2%	2.63e-06	2.06	0.0389	0.464	-14.77	0.460	0.342
Std	2.74e-6	4.97e-16	6.94e-18	5.722e-17	5.1984e-5	3.34e-16	7.85e-17
R^2	0.999996	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000

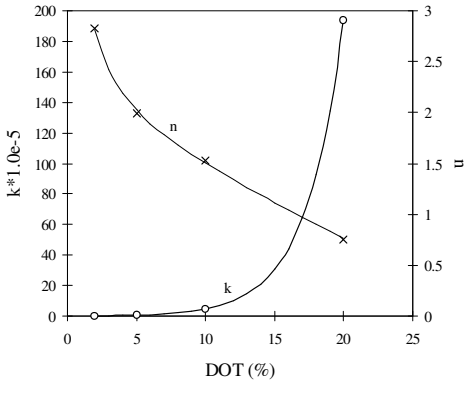
$m_s = 0.00412$

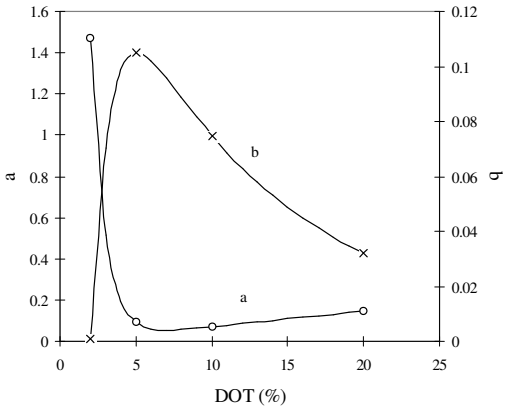
Table 2. Influence of DOT on the kinetic parameters of Model 2.

DOT	k	n	a	b	c	d	Yx/s	Yp/s
20%	1.94e-03	0.75049	0.14644	0.032088	0.056626	9.4729	0.33557	0.47189
10%	4.94e-05	1.5341	0.071946	0.074943	0.098265	9.6389	0.24962	0.47625
5%	5.34e-06	1.9949	0.091397	0.10488	0.082206	9.5441	0.42183	0.31412
2%	6.21e-08	2.8258	1.4685	7.58E-04	0.062257	8.2343	0.83932	0.31985
Std	1.79e-10	0.031607	3.235e-4	4.19e-17	6.135e-4	2.00e-03	5.70e-05	4.37e-5
R^2	0.999998	0.999116	0.9999998	1.000000	0.9996556	0.999994	1.000000	1.000000

Table 3. Statistical analysis of the model prediction

		$kS^n+Rp(4)$		$kS^n+Rp(3)$	
		Std	R^2	Std	R^2
20%	Biomass	0.565	0.9973	0.6262	0.9968
	Lysine	0.8814	0.9986	1.0210	0.9986
	Glucose	3.7326	0.9984	3.7586	0.9984
10%	Biomass	0.9212	0.9916	0.9255	0.9884
	Lysine	1.5776	0.9988	1.6731	0.9970
	Glucose	2.1193	0.9993	4.9572	0.9983
5%	Biomass	1.2624	0.9553	1.3843	0.9608
	Lysine	0.7668	0.9984	1.1546	0.9984
	Glucose	3.1156	0.998	5.3339	0.9966
2%	Biomass	0.955	0.9789	1.1850	0.9700
	Lysine	0.8417	0.9988	1.5938	0.9951
	Glucose	1.4083	0.9996	4.5404	0.9965





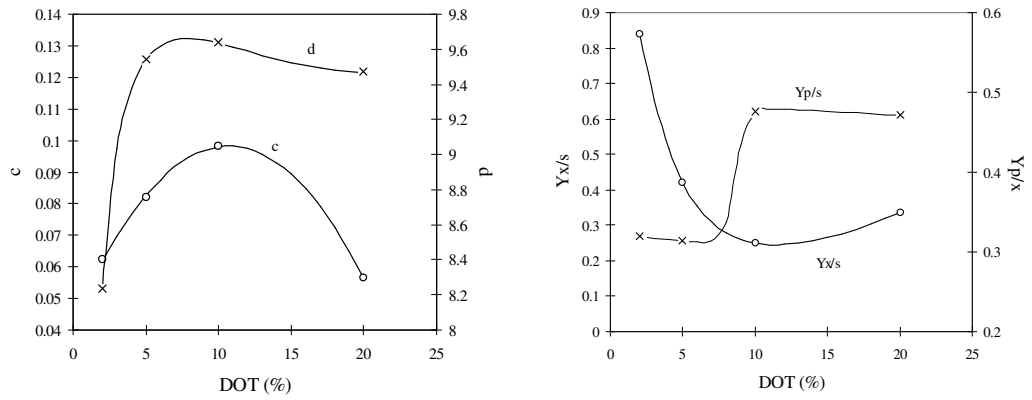


Figure 5. Correlation of DOT [2 20] to the kinetic parameters of Model 2.

k