EXPERIMENTAL DESIGN TECHNIQUES APPLIED TO STUDY OF OXYGEN CONSUMPTION IN A FERMENTER[1]

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Abstract: *The dependence of the volumetric rate of oxygen consumption on the variables agitation and air flow, was studied in a laboratory scale fermenter. A 2^2 factorial experimental design, with four axial points and four replicates of the central point, was used. The coefficients for the two-variables, quadratic model were calculated. The 'fit' of the model with the empirical data was studied using a lack of fit test. The surface response methodology was used to maximize the dependent variable, volumetric rate of oxygen consumption. The response surface obtained showed an absolute maximum on the domain's boundary, consistent with theoretical considerations indicating that a relative maximum inside the domain was impossible. This approach allowed us to derive a model to predict volumetric rate of oxygen consumption in a standard laboratory fermenter, as a function of agitation and air flow, i.e., the two variables selected.*

Key words: *surface response methodology; volumetric rate of oxygen consumption*

Introduction

Production of recombinant proteins is most economical when there is a high cell concentration in the fermenter and the protein product is also expressed at a high level. Aerobic or facultative anaerobic cells in culture require adequate transfer of oxygen from the gaseous phase (usually air, or air enriched with oxygen) to the liquid phase, where the cells consume oxygen for respiration. In lab experiments with flasks, oxygen supply is typically achieved by flask stirring with a high ratio of air to liquid flask volume, which often becomes a significant parameter (Martínez *et al.*, 2006). Aeration and agitation are carried out in the fermenter to provide the culture with oxygen, and to some extent to stabilize physicochemical parameters. In aerobic fermentations, oxygen supply may become a limiting factor, especially in very large-scale systems. Oxygen is not very soluble (its saturation concentration in water is 7 mg L-1 when air is bubbled through it at 1 atm. at 30°C) (Bailey and Ollis, 1986). Its rate of solubilization is a function of several factors: the area of the gas-liquid interphase, temperature, contact time, composition of the culture medium, cell density and cell activity, among others (Pirt, 1975).

In a given system, some factors, such as temperature, are pre-set or are easily controlled in order to achieve optimum conditions. Therefore, the contact area, the contact time, and agitation are the most important variables determining oxygen transfer (Werman and Wilki, 1973). Oxygen supplied into the fermenter by air flow and agitation becomes limited as time goes on, due to alterations in the medium. There are therefore two separate factors that often determine how long the culture can continue. The first factor is the demand for oxygen, which requires more or less oxygen in solution in the culture medium, according to the requirements of the cells. The second factor is the capacity of the system to transfer oxygen into the liquid phase. Demand for oxygen in the culture is determined by cell concentration, the specific growth rate, and oxygen transfer into the medium, and is satisfied by oxygen in the culture medium entering the cells (Calam and Russell, 1973). Oxygen transfer is limited by the velocity of air bubbles through the culture medium, and the velocity of oxygen transport into cells. It is important to use appropriate experimental design to discover the oxygen transfer potential of the fermenter, according to its operating conditions (Phillips and Johnson, 1961).

In our work, we have studied the variation in volumetric rate of oxygen consumption ($k_L a C_L^*$) of the culture as a function of agitation and air flow. From a statistical point of view, this implies studying a real function with two independent variables, preferably using the surface response methodology (S.R.M.) (Montgomery, 2005). We used a linear regression model with two variables, and the following terms: an independent term, two linear terms, two pure quadratic terms, and an interaction term. The main reason for choosing this model was our interest in studying two effects: curvature and interaction between variables. In order to adjust the parameters of this model to our data, the experiment was properly designed with replicate data points so as to be able to estimate error. We chose the Box-Wilson design (Box and Wilson, 1951), commonly called central composite design (CCD), in our case with four centerpoint runs (Montgomery, 2005).

Our aim was to obtain a well-validated quadratic model which would allow prediction of $k_L a C_L^*$ values, in a fermenter, as a function of air flow and agitation, within the working limits of those variables. This approach should be useful for the future evaluation of recombinant streptolysin-O (Velázquez *et al.*, 2005) production in a fermenter.

Materials and methods

Determination of the volumetric rate of oxygen consumption. A 3-litre fermenter (Bio Flo III, Batch-Continuous Fermenter, New Brunswick Scientific, Edison, N.J., U.S.A.) was used with a working volume of 2 litres of water. The fermenter was automatically controlled by software (AFS-BioCommand Bioprocessing Software, version 2.60; New Brunswick Scientific). Volumetric rate of oxygen consumption ($k_L a C_L$*) was determined by oxidation of $S_2O_3^{2-}$ to $SO_4^{\ 2-}$ in the presence of metal ions (Co $^{2+}$ or Cu $^{2+}$) as the catalyst (Wernan and Wilki, 1973). The determination was carried out in 2 litres of distilled water at 37°C, with 20 g/L $S_2O_3^{2}$- and 1.5 g/L $CuSO_4$ as catalyst. For each assay, the rotation speed of the agitator was adjusted, and air flow was also adjusted, according to the settings pre-determined by the experimental design (50 - 450 r.p.m. for agitation and 0.500 – 4.000 L/min. for air flow). Samples of 5 mL were taken for analysis at different time-points (from t = 0 and at intervals of 5 to 15 minutes) until all the sulfite had been oxidized.

Experimental Design. With the volumetric rate of oxygen consumption ($k_L a C_L$*) results, surface response studies were carried out using a 2^2 factorial experimental design, with four axial points (($\pm\alpha$, 0) and (0, $\pm\alpha$), where $\alpha = \sqrt{2}$) and four replicates of the central point, totaling 12 experiments (Montgomery, 2005). The coded values of the independent variables were: -α, 1, 0 (central point), +1, +α (Table 1). The experimental plan is shown in Table 2 for the coded values in Table 1.

Table 1. Coded values of independent variables. Coded levels used in experimental design for S.R.M. of $k_L a C_L$* in fermenter.

Variable	Symbol	Levels				
		-α	-1	0	+1	+α
Agitation (r.p.m.)	X_1	50	109	250	392	450
Air flow (L/min.)	X_2	0.500	0.836	2.250	3.660	4.000

Table 2. Experimental plan for SRM study of $k_L a C_L$*. Values of independent variables agitation (r.p.m) and air flow (L/min.) for determining $k_L a C_L$* in the fermenter, coded as in Table 1.

Experiment N°	Agitation (x_1)	Air flow (x_2)
1	-1	-1
2	+1	-1
3	-1	+1
4	+1	+1
5	0	0
6	0	0
7	-α	0
8	+α	0
9	0	-α
10	0	+α
11	0	0
12	0	0

Analysis of the proposed model. Analysis of the model proposed for the observed data, and calculation of its coefficients, were carried out using the Statistica version 4.5 software (Statsoft). For preliminary assessment of the model's fit, correlation coefficient R and its square, R^2, were used (Montgomery, 2005). The statistical significance of the parameters of the model was determined by Student's *t* test and corroborated by Fisher's *F* test (Montgomery, 2005). In this test, the higher the critical value of *t* and the lower the value of *p*, the greater the effect or significance of the coefficient in the model (Montgomery, 2005). Subsequently, the goodness of fit of the model was examined by the lack of fit (L.O.F.) test (Draper and Smith, 1998). There were 5 degrees of freedom in our full quadratic model. Twelve experiments were carried out, with n-1 degrees of freedom. The degrees of freedom of the error were calculated by difference as always. Finally, the surface response methodology (Montgomery, 2005) was used to find the maximum volumetric rate of oxygen consumption ($k_L a C_L$*).

Results

Determination of the coefficients for the model of volumetric rate of oxygen consumption ($k_L a C_L$*) as a function of agitation and air flow. Table 3 shows experimental values for volumetric rate of oxygen consumption, and those calculated according to the following quadratic model:

$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$

where y is the predicted value of volumetric rate of oxygen consumption ($k_L a C_L$*) (mmol/L/h)

X_1= agitation (r.p.m.) in coded form

X_2= air flow (L/min.) in coded form

$y = 15.50014 + 22.58148 X_1 + 6.43198 X_2 + 13.12500 X_1^2 - .87500 X_2^2 + 4.00000 X_1X_2$.

Table 3. Comparison of experimental and calculated values of $k_L a C_L$*. Experimental values of $k_L a C_L$* were obtained by the sulfite oxidation method. Calculated values of $k_L a C_L$* were obtained from the regression equation $y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1X_2$

Agitation (r.p.m.)	Air flow (L/min.)	Experimental $k_L a C_L$* (mmol /L/h)	Calculated $k_L a C_L$ * (mmol /L/h)
109	0.836	5.00 +/- 0.08	2.74
392	0.836	35.00 +/- 0.87	39.90
109	3.660	10.00 +/- 0.15	7.60
392	3.660	56.00 +/- 1.87	60.76
250	2.250	12.00 +/- 0.01	15.50
250	2.250	16.00 +/- 0.27	15.50
50	2.250	6.00 +/- 0.10	9.81
450	2.250	80.00 +/- 4.00	73.68
250	0.500	6.00 +/- 3.00	4.65
250	4.000	24.00 +/- 0.61	22.85
250	2.250	17.00 +/- 0.28	15.50
250	2.250	17.00 +/- 0.28	15.50

Correlation and parameter significance. Results showed that the model fitted with the experimental values in the ranges of the variables studied. Multiple regression analysis results were R = 0.9885 and R^2 = 0.9770, indicating a high degree of correlation between the experimental values and those predicted by the model.

Table 4 shows Student's *t* test applied to individual coefficients in the model, to test their significance. Coefficients β_1, β_2 and β_{11} were statistically significant at a 95% confidence level. These are the coefficients for the linear terms for air flow and agitation, and for the quadratic term of agitation.

Table 4. Linear and quadratic coefficients estimated for the quadratic model. Estimated values and probability levels according to Student´s *t* test.

Coefficient	Effect on model	Student´s *t* (5 d.f.)	Probability level (p)
β_1	22.58148	13.56670	.000039
β_2	6.43198	3.86426	.011829
β_{11}	13.12500	7.05288.	.000886
β_{22}	-.87500	-.47019	.658013
β_{12}	4.00000	1.69929	.150014

Table 5 shows analysis of variance (ANOVA) for Fisher's *F* test. The calculated value of *F* was greater than the tabulated value ($F_{1,5}$ = 6.61), in the case of the coefficients β_1, β_2 and β_{11}, being significant at the 95% confidence level. However, the *F* values for coefficients β_{22} and β_{12} were not significant. It can be concluded from these results that agitation is the most significant factor in determining volumetric rate of oxygen consumption, followed by the quadratic term for agitation, and thirdly by the air flow.

Table 5. Analysis of variance (ANOVA) for regression model of $k_L a C_L^*$ as a function of agitation and air flow. Significance of the model coefficients (NS: not significance at 95 %, S: significance at 95%).

Source of variation	Sum of squares	Degrees of Freedom	Mean squares	*F*	p-value	Signifi-cance level
β_1	4079.384	1	4079.384	184.0555	.000039	S
β_2	330.963	1	330.963	14.9325	.011829	S
β_{11}	1102.500	1	1102.500	49.7431	.000886	S
β_{22}	4.900	1	4.900	.2211	.658013	NS
β_{12}	64.000	1	64.000	2.8876	.150014	NS
Residual	110.819	5	22.164			

Goodness of fit and response surface of the model. A lack of fit (L.O.F) test was performed and the results are shown in Table 6.

Table 6. ANOVA for L.O.F. test.

Item	Degrees of Freedom	Sum of squares	Mean squares
Model	5	5658.50775	1131.70155
Error	6	132.15892	22.02649
Total	11	5790.66667	

A statistic value $F = 51.38$ can be obtained by dividing mean squares of model by mean squares of error (Table 6). This value was compared with the critical region $[8.75, +\infty)$, obtained by using the F function table, with 5 degrees of freedom for the model and 6 degrees of freedom for the error, with a significance level of $\alpha = 0.01$. Thus, the 'fit' of the model was statistically significant at the 99% confidence level.

The surface response methodology applied to the experimental conditions tested identified a maximum volumetric rate of oxygen consumption of 89.88 mmol/L/h. This maximum corresponded to the coded values of 1.5 for agitation and air flow, that is, 462 r.p.m. and 4.100 L/min. respectively. Thus the maximum is achieved at the highest values of both variables, within the experimental ranges (450 r.p.m and 4.000 L/min. respectively) (Figure 1).

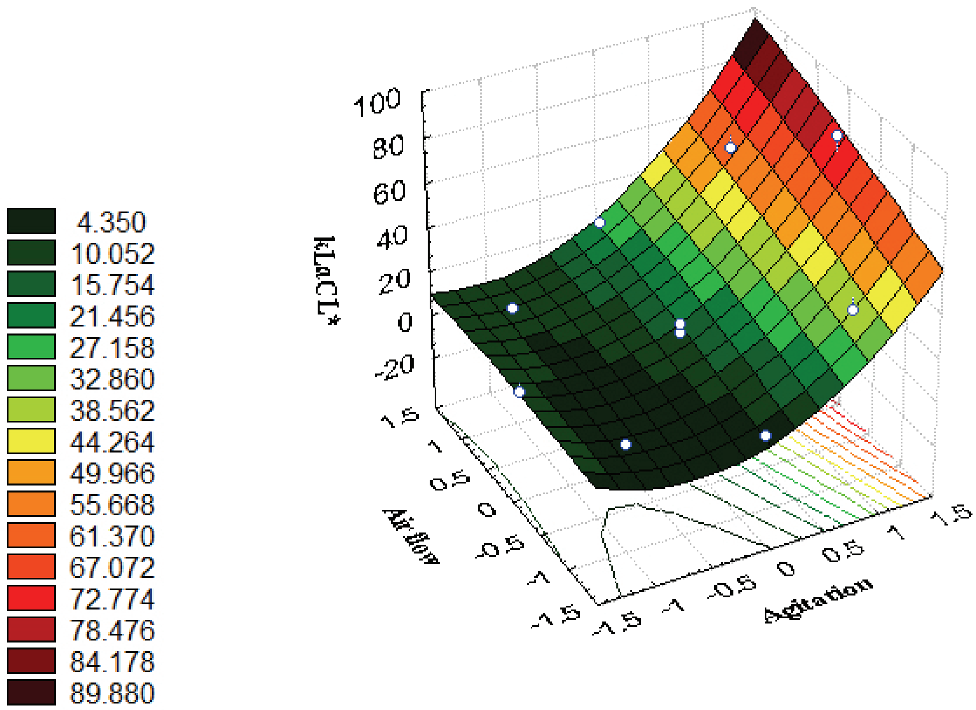

Figure 1. S.R.M. for $k_L aC_L$* values. $k_L aC_L$* as a function of agitation and air flow (coded values as in Table 1).

Discussion and conclusions

The experimental design chosen was appropriate for estimating the coefficients in a full quadratic regression with two independent variables. In our particular case, we concluded that the 'fit' of the model to the experimental data was statistically significant at the 99% confidence level. However, some of the individual coefficients of the model equation were not significant, even at the 95% confidence level. The non significant coefficients were: the quadratic term for air flow (β_{22}) and the one for the interaction between agitation and air flow (β_{12}). The implication from the bioengineering point of view is that the most important variable to take into account in ensuring adequate volumetric rate of oxygen consumption ($k_L aC_L$*) in cultures in a fermenter, is agitation. However, the biological needs of the microorganism must be met in order to maximize the yield of the desired recombinant protein, so air flow must also be taken into account, as a complementary variable. From the statistical point of view, the above could suggest applying a significance test for a sub-model (Rencher, 2000), in order to examine 'goodness of fit' with a simpler model in which the quadratic term for air flow and the interaction term are excluded.

The obtained surface corresponding to the full quadratic model might be further explored by increasing the experimental values of both air flow and agitation.

The absolute maximum for volumetric rate of oxygen consumption that we found was located at the vertex of the region, corresponding to the highest values of the variables. Since the function $k_L a C_L^*$ increases with respect to both air flow and to agitation, a relative maximum inside the domain cannot be expected (Banks, 1980). The results of the S.R.M. performed (Figure 1) are totally consistent with the above theoretical consideration. If the same procedure is carried out to search for another maximum, using higher values for air flow and agitation, we would expect a surface response similar to Figure 1, i.e., with the function increasing with both variables, and an absolute maximum at the boundary of the domain. It would be convenient to use a maximum slope method for this exploration (Montgomery, 2005). For this purpose, determining the maximum slope as a function of the variables of the model would be useful.

References

1. Bailey, J. E. and Ollis, D. F., **Biochemical engineering fundamentals**; 457-500. 2nd Edition, McGraw-Hill Book Co. New York, U.S.A, 1986
2. Banks, G. T. , **Scale-up of fermentation process,** Top. Enzyme Ferment. Biotechnol. 3, 170-266, 1980
3. Box, G. E. and Wilson, K. B., **On the Experimental Attainment of Optimum Conditions**, Journal of the Royal Statistical Society, Series B, 13, 1-45, 1951
4. Calam, C. T. and Russell, D. W., **Microbial aspects of fermentation process development,** J. Appl. Chem. Biotechnol. 23, 225-237, 1973
5. Draper, N. R. and Smith, H., **Applied Regression Analysis,** 47-55. J Wiley & Sons, New York, U.S.A, 1998
6. Martínez, V., Guineo, G., Velázquez, B., Chabalgoity, J. A. and Massaldi, H., **Bifactorial design applied recombinant protein expressions**, Journal Data Science, 4, 247-255, 2006
7. Montgomery, D. C., **Design and Analysis of Experiments;** 405-458. 6th Edition, J. Wiley & Sons. New Jersey, U.S.A, 2005
8. Phillips, D. H. and Johnson, M. J., **Oxygen transfer in fermentations,** Sci. Rep. Ist. Super. Sanita, 1, 1 90-195, 1961
9. Pirt, S. J., **Principles of Microbe and Cell cultivation,** 4-15. 1st. Edition, Blackwell Scientific Publications, London, U.K, 1975
10. Rencher, A. C., **Linear Models in Statistics,** 170-213. John Wiley & Sons, New York, U.S.A, 2000
11. Velázquez, B., Massaldi, H., Battistoni, J. and Chabalgoity, J. A., **Construction and expression of recombinant Streptolysin-O; pre-evaluations of it use in immunoassays,** Clinical Laboratory Diagnostic Immunology, 12, 683-684, 2005
12. Wernan, W. C. and Wilki, C. R., **New method for evaluation of dissolved oxygen response for Kla determination,** Biotech. Bioeng.,15, 571, 1973

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