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Applying Modern Design of Experiments to Mass Transfer Characterization in Bioreactors

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Mass transfer is one of the most crucial parameters during scale-up in cell culture, and may cause variations in specific yields. At the cellular level, all the cells require proper supplementation of essential nutrient sources: nitrogen, oxygen, and carbon. The ability of cells to grow, maintain viability, and provide high specific productivity depends on the proper distribution of these nutrients. The latter depends on proper mass transfer to supply oxygen, carbon, and nitrogen through agitation and aeration, along with other micronutrients.

Numerous articles have already discussed the importance of mass transfer and its dependence on agitator power per liquid volume. While these studies describe effective ways of characterizing and overcoming mass transfer limitations, they fail to use a systematic approach to report statistical significance of their results. In this study, statistical design of experiments (DOEs) are used to assess the suitability of the equipment design during scale-up in bioreactors.

Mass Transfer in Bioreactors

There are three phenomena in an aerated tank that dominate the biologicals production: 1) thermodynamics; 2) microbial kinetics; and 3) transport phenomena. Of these, thermodynamics and microbial kinetics are independent on scale, but the transport phenomena (comprised of mixing, mass and heat transfer) is scale-dependent. Thus, the primary variables under scrutiny are baffles, impeller type, mass transfer

coefficient, power number, density, tank diameter, and stirrer speed. The mass transfer coefficient for each species (*i.e.*, oxygen, carbon dioxide) is calculated using its respective mass balance and it is correlated to agitator power and gas flow rate as given below:

$$\frac{dO_2}{dt} = k_{LO}a (O_2^* - O_2) \quad (1)$$

$$\frac{dCO_2}{dt} = k_{LC}a (CO_2^* - CO_2) \quad (2)$$

$$k_La = A \left(\frac{P_g}{V} \right)^B (U_s)^C \quad (3)$$

Where O_2^* and CO_2^* are, respectively, the concentration of dissolved oxygen (dO_2) and dissolved carbon dioxide (dCO_2) in the liquid at saturation in mg/L, O_2 and CO_2 represent the concentration of dO_2 and dCO_2 in liquid, mg/L, and $k_{LO}a$ and $k_{LC}a$ are the mass transfer coefficients of oxygen and carbon dioxide in s^{-1} .

In equation 3, k_La is overall mass transfer coefficient in s^{-1} , A is the conversion constant, B and C are dimensionless empirical constants, P_g is the power required from the agitator to achieve a desired concentration of dissolved oxygen in kW, V is the liquid volume in m^3 , and U_s is the superficial gas velocity in m/s. In cell culture, stripping of excess carbon dioxide is critical for optimal production.^[1] Therefore, equation 2 can be simplified by eliminating carbon dioxide in the influent gas (consequently, CO_2^* is zero).

$$\frac{dCO_2}{dt} = -k_{LC}a CO_2 \quad (4)$$

Where $k_{LC}a$ can be referred to as carbon dioxide stripping coefficient. If sufficient data is available, proper impeller with required agitator can be designed. Currently, the most common

bioreactors use either the radial-flow (*i.e.*, Rushton turbine) or axial-flow impellers, for which the power-draw characteristics are almost never published.^[2] Thus, the impeller-power under gassed conditions are calculated using specific correlations dependent upon specific impeller designs.

$$N_p = P_u / \rho N^3 D^5 \quad (5)$$

$$P_g / P_u = f(N_a, D/T, N_{Re}, N_{Fr}) \quad (6)$$

Where N_p is the dimensionless impeller power number, P_u and P_g are the ungassed and gassed agitator power in kW, ρ is the liquid density in kg/m^3 , N is agitator-shaft rotational speed in s^{-1} , D is the impeller diameter in m, and f is a function of the dimensionless aeration number (N_a), ratio of impeller diameter-to-tank inside diameter (D/T), and dimensionless Reynolds and Froude number (N_{Re} , N_{Fr}).

The power under gassed conditions is calculated first by calculating the ungassed power using equation 5, and then calculating the gassing factor from equation 6 using a function of N_a ($= Q_g / ND^3$), D/T , and N_{Re} and N_{Fr} which varies with various impeller types.^[2] As a consequence, the power calculations provided by the manufacturer often depend on the assumptions made by the individual manufacturers; and therefore, the accurate prediction of power is still unattainable.^[3]

While bioreactors are typically designed using calculations the designer considers appropriate, end-users often encounter scale-up issues when optimizing their processes. The process optimization mainly involves the feed profiles, media composition, and process parameters such as pH, temperature, and dissolved oxygen set points. Sometimes scale-up issues may be independent of

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these parameters. One variable often overlooked is the liquid volumetric mass transfer coefficient which is highly dependent on the equipment design and has an effect on the dispersion of the gas through the liquid. The latter is used for characterizing oxygen transfer and can be very advantageous if it is determined accurately based on data rather than assumptions.^[4] Furthermore, the gas influent rate is critical in providing sufficient dissolved oxygen. A lower flow rate could cause lack of dissolved oxygen in the liquid bulk, while a high flow rate could cause flooding.

The definition of flooding is not well-defined, but it is often used in bioreactor engineering as a term to describe the improper stirring in a tank or the appearance of ragged cavities behind the blades of the individual impellers (impeller-flooding). With dual and triple radial-flow impellers, the flooding occurs at the lowest impeller with low gas flow, and continues to the middle and top impellers as the gas flow increases.^[5] While ignored at times, the issue of flooding can be resolved using the statistical methods presented in this study.

In cell culture, providing oxygen in the form of gas bubbles through sparger can be detrimental to the cells. The interaction of bubbles with the cells can cause shear stress on the cells and damage the suspended cells. The most common mechanisms of cell-bubble damage are: 1) bubble generation at the sparger; 2) rising bubbles; 3) bubbles coalescing and breaking up; and 4) bubbles at the air-medium interface. Furthermore, the varied sizes of the air bubbles can cause improper oxygen transfer.^[6-7]

Carbon dioxide stripping is another important aspect of bioreactor and cell culture and is defined as the rate of carbon dioxide removed from liquid bulk to maintain constant levels of dissolved carbon dioxide. As the cell concentration increases, carbon dioxide can accumulate to toxic or metabolism-altering levels if the carbon dioxide balance is not maintained correctly between the head space of the bioreactor and the liquid bulk.^[8]

Although the issues just described have been extensively studied using deterministic models and correlations, the use of statistical design of experiments is limited. Present research investigates the oxygen transfer and carbon dioxide stripping in bioreactor engineering and cell culture through statistical models using I-optimal and D-optimal designs.

Modern Design of Experiments

While any model is just an approximation of reality, models based on first principles are the best when one can define the problem using fundamental equations such as conservation of mass, energy, and momentum. Alternatively, statistical models provide efficient and effective solutions to the real-life problems without defining any fundamental processes. DOEs provide statistical methods to attain time- and cost-effective solutions either to discover the sources of variation in a measured response or to compare values of the response at different settings of controllable variables.^[9]

Classical design of experiments includes full factorial, fractional factorial, and response surface designs that require large numbers of runs to reveal interactions between factors. While these designs are balanced and orthogonal, in practice, many applications deal with irregular experimental regions, nonstandard models, and unusual samples or block sizes. Therefore, modern DOEs became more appropriate than classical designs

in dealing with some industrial applications. These designs are characterized by letters of the alphabet and are often called alphabetic optimality criteria. The best known and most often used criterion is D-optimality.

These designs are good for screening experiments because they focus on precise estimates of the coefficients. The experimenter can tell which factors' effects are important and which are negligible. Thus, in simple words, this design is good if an experimenter is trying to narrow down the factors and remove the irrelevant ones. While D-optimal designs minimize the variance of parameter estimates, I-optimal designs minimize the average prediction variance inside the region of the factors. Therefore, I-optimal designs are useful for prediction. They tend to place fewer runs at the extremes of the design space than does a D-optimal design. As a result, D-optimal designs often predict better at the extreme values of the factors.

Figure 1 depicts the one-factor quadratic response surface design using both D-optimality and I-optimality criteria for 12 runs. As expected, the prediction variance for the D-optimal design is uniform within the region of interest. On the contrary, the I-optimal design has the lowest prediction variance at the center (more center points in this design). The D-optimal design is able to predict variance at the center of the factor space and is almost 1.5 times the variance of the I-optimal design.

FIGURE 1. D-optimal design minimizes variance of parameter estimates and I-optimal minimizes average variance.

Materials and Methods

Bioreactor Operating Conditions:

A BIOSTAT®A plus bioreactor control unit (Sartorius-Stedim Biotech, Goettingen, Germany) was used for parameter controls. A 7 L bioreactor (4 L working volume, H/D = 1.2:1) was used for Study 1 and it was operated with varying temperatures, gas sparging rates, and agitation speed. A 20 L bioreactor (Applikon Biotechnology, Foster City, California) was used for Study 2 (18 L working volume, H/D = 2.2:1). (See Table 1.)

While the temperature and gas sparging rate was maintained at 37°C and 0.025 vvm, respectively, agitation was investigated at two levels in the 20 L bioreactor. They were equipped with condensers to minimize any evaporative losses due to off-gassing. The mixed media containing 75% standard DMEM (Invitrogen Corp., Carlsbad, CA), and 25% Terrific Broth (Mediatech, Inc., Manassas, VA) was used to emulate semi-defined media conditions. The foaming inside the bioreactor was controlled using P2000 propylene

glycol (Dow Chemical, Midland, MI) at 2 mL/L. The sterility of the media was maintained using an antibiotic.

Determination of Mass Transfer Coefficients

The mass transfer coefficients are calculated using equations 1 and 4 (discussed earlier). Equations 7 and 8 are derived by integrating equations 1 and 4.

$$\ln \left(\frac{O_2^* - O_2^0}{O_2^* - O_2} \right) = k_{LO}at \quad (7)$$

$$\ln \left(\frac{CO_2^0}{CO_2} \right) = k_{LC}at \quad (8)$$

Where O_2^0 and CO_2^0 are the initial concentrations, and O_2 and CO_2 are the oxygen and carbon dioxide concentrations in liquid at time t , respectively. The variation in carbon dioxide is directly related to the variation in pH values. Therefore, equation 8 can be rewritten as:

$$(pH - pH^0) = k_{LC}at \quad (9)$$

The time profile of both pH and dissolved oxygen was recorded and

mass transfer coefficients, k_{LO} and k_{LC} , were calculated from the slope of time profiles. Initially, the bioreactors were sparged with nitrogen to strip all oxygen in the liquid. Subsequently, the vessels were sparged with carbon dioxide to lower pH. Both carbon dioxide stripping and oxygen transfer were measured when sparging with air.

Study 1, D-Optimal Design:

The factors under investigation for this primary study were agitation speed, impeller type, media type, temperature, sparger type, and aeration. The agitation speeds were tested at 75 and 675 rpm. The temperature was tested at 25 and 37°C. The impeller types selected for the experimentation were pitched and Rushton, and the specifications are provided in Table 1. Standard DMEM and DMEM with yeast extract were used for testing media types. The aeration was set to 0.025 and 0.075 vvm for testing the effects of sparging air. Both micro and two-hole spargers were used to observe the effects of sparger types on dissolved oxygen and carbon dioxide.

TABLE 1. Bioreactor specifications.

TABLE 1. Bioreactor specifications.				
Parameters	7 L Sartorius		20 L Applikon	
Working volume (L)	4.0		18.0	
Tank diameter (cm)	16.0		22.3	
H/D	1.2		2.2	
Impeller types	Pitched	Rushton	Pitched	Rushton
Impeller diameter (cm)	7.4	7.0	10.0	7.6
Number of impellers	1.0	1.0	2.0	2.0
Ring sparger number of holes	2.000		3.000	
Ring sparger hole diameter (mm)	0.020		1.000	
Micro-sparger surface area (cm ³)	0.785		1.000	
NOTES: Rushton impellers: Sartorius-Stedim; pitched-blade impellers: Applikon. Spargers (components of each bioreactor vessel): 7 L, Sartorius-Stedim; 20 L, Applikon.				

Study 2, I-Optimal Design:

On the basis of the Study 1 results, agitation speed, impeller type, and sparger type were further investigated in this experiment using the I-optimal design. Agitation speeds of 60 and 800 rpm were used, respectively. Both micro and three-hole spargers were further investigated in this study. The pitched and Rushton were the two impeller types selected for this study.

Results and Discussion

The effects of six different parameters (agitation speed, impeller type, media type, temperature, sparger

type, and aeration) on the mass transfer coefficient of oxygen and carbon dioxide stripping were studied in bioreactors using response surface designs. The results are reported in the form of interaction profiles where separated lines indicate main effects and the difference in slopes suggest interaction between parameters. The statistical analyses for all designs were conducted at a significance level of 0.05. The experimental data satisfied three basic assumptions of analysis of variance tests (ANOVA) such that the error is independent, normally distributed, and has constant variance.

Study 1, D-Optimal Design:

The interaction profiles for the response surface design are depicted in Figure 2, with mass transfer coefficient for oxygen as the response variable. In the interaction profile, agitation rate had the main effect on the mass transfer coefficient for oxygen as observed by large separation of lines across other parameters. There was significant slope difference in the interaction profile of the oxygen mass transfer with sparger type and agitation. These results are confirmed by the effect test. As shown in Table 2, the agitation speeds are crucial for appropriate dispersion of oxygen in a stir-tank reactor ($p < 0.05$).

FIGURE 2. Interaction profile for the Study Design 1 using D-optimal model. The y-axis reads the mass transfer coefficient for oxygen, and the x-axis represents the various parameters using two agitation speeds (75 and 675 rpm).

TABLE 2. The effect tests and significance of the different parameters on the oxygen mass transfer in Study 1.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Impeller type	1	1	22.7437	0.1050	0.7496
Media type	1	1	21.3744	0.0987	0.7570
Temperature (25,37)	1	1	0.3549	0.0016	0.9682
Sparger type	1	1	603.2940	2.7865	0.1124
Agitation (75,675)	1	1	3539.8119	16.3497	0.0008
Aeration (100,300)	1	1	152.4171	0.7040	0.4125
Impeller type/Media type	1	1	617.1988	2.8507	0.1086
Impeller type/Temperature	1	1	33.4153	0.1543	0.6990
Impeller type/Sparger type	1	1	973.6953	4.4973	0.0481
Impeller type/Agitation	1	1	279.5903	1.2914	0.2707
Impeller type/Aeration	1	1	76.2736	0.3523	0.5602
Media type/Temperature	1	1	378.8403	1.7498	0.2025
Media type/Sparger type	1	1	392.1891	1.8114	0.1950
Media type/Agitation	1	1	77.7006	0.3589	0.5566
Media type/Aeration	1	1	2.5470	0.0118	0.9148
Temperature/Sparger type	1	1	16.1085	0.0744	0.7881
Temperature/Agitation	1	1	8.1184	0.0375	0.8486
Temperature/Aeration	1	1	9.4634	0.0437	0.8367
Sparger type/Agitation	1	1	1521.9748	7.0297	0.0162
Sparger type/Aeration	1	1	102.0735	0.4715	0.5011
Agitation/Aeration	1	1	295.3544	1.3642	0.2581

The interaction between the impeller type and sparger type, as well as the interaction between the agitation speed and sparger type, were also found to be significant ($p < 0.05$).

While carbon dioxide stripping was also investigated using the same parameters, there was no statistical significance with any main effects and interactions (data not shown). This was due to the fact that the carbon dioxide stripping within a small-scale vessel is very efficient, and thus there is no apparent impact on the stripping of carbon dioxide.

Study 2, I-Optimal Design:

The prediction profile and the effects tests for the response surface design are depicted in Figure 3 and Table 3 with mass transfer coefficient for oxygen as the response variable. Agitation and impeller type were found to have a significant effect on the oxygen mass transfer coefficient at maximum agitation (depicted by red-dotted lines) as portrayed in the prediction profile (Figure 3). The latter results are confirmed by the effect tests in Table 3 for agitation and impeller type ($p < 0.05$).

The prediction profile and the effects tests for the response surface design are depicted in Figure 4 and Table 4 with the carbon dioxide stripping coefficient as the response variable. While both the sparger and impeller types did not play a significant role, the agitation showed both main and second-order effects on the carbon dioxide stripping ($p < 0.05$).

Conclusion

Mass transfer characterization is crucial during the scale-up of both cell culture and microbial processes. Modern design of experiments provides statistically significant solutions that eliminate the use of empirical correlations. Two crucial parameters evaluated in this study were mass transfer coefficient of oxygen and stripping coefficient of carbon dioxide, which are dependent on agitation speed, impeller type, media type, temperature, sparger type, and aeration.

FIGURE 3. Prediction profile for the mass transfer coefficient of oxygen against the three parameters in Study 2.

TABLE 3. The effect tests and significance of the different parameters on the oxygen mass transfer in Study 2.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Agitation (60, 800)	1	1	2071.2013	110.3513	<0.0001
Sparger type	1	1	0.0457	0.0024	0.9619
Impeller type	1	1	139.0336	7.4076	0.0262
Agitation/Agitation	1	1	1.1583	0.0617	0.8101
Agitation/Sparger type	1	1	24.8336	1.3231	0.2833
Agitation/Impeller type	1	1	239.2250	12.7456	0.0073
Sparger type/Impeller type	1	1	30.9442	1.6487	0.2351

FIGURE 4. Prediction profile for the stripping coefficient of carbon dioxide against the three parameters in Study 2.

TABLE 4. The effect tests and significance of the different parameters on the carbon dioxide mass transfer in Study 2.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Agitation (60, 800)	1	1	0.32200313	62.7337	<0.0001
Sparger type	1	1	0.00532900	1.0382	0.3381
Impeller type	1	1	0.00748225	1.4577	0.2618
Agitation/Agitation	1	1	0.06076225	11.8379	0.0088
Agitation/Sparger type	1	1	0.00973012	1.8957	0.2059
Agitation/Impeller type	1	1	0.00195312	0.3805	0.5545
Sparger type/Impeller type	1	1	0.00950625	1.8520	0.2106

The experimental design used in Study 1 revealed that impeller type, sparger type, and agitation speed were the most important factors in mass transfer characterization. Further experimental design using different

vessel sizes showed an interaction between agitation and impeller type. The Rushton impeller demonstrated better oxygen mass transfer than pitched-blade impeller at maximum agitation. On the other hand, this

difference was unimportant at low agitation. Therefore, the results suggest that at low agitation speeds, standard gas flow rates may lead to flooding of the impellers and limit effective oxygen mass transfer.

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