Optimization of Cultivation Conditions in Spin Tubes for Chinese Hamster Ovary Cells Producing Erythropoietin and the Comparison of Glycosylation Patterns in Different Cultivation Vessels

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DOI 10.1002/btpr.390
Published online January 29, 2010 in Wiley InterScience (www.interscience.wiley.com).

This article describes the optimization of cultivation factor settings, that is the shaking rate and working volume in 50 mL spin tubes for a Chinese hamster ovary cell line expressing recombinant human α-erythropoietin, using a response D-optimal surface method. The main objectives of the research were, firstly, to determine a setting in which the product titer and product quality attributes in spin tubes are equivalent to those in 250 mL shake flasks in a seven day batch and, secondly, to find a setting in which the product titer is maximal. The model for product titer prediction as a function of shaking rate and working volume in the defined design space was successfully applied to the optimization of cultivation conditions in spin tubes for the tested cell line. Subsequently, validation experiments were carried out simultaneously in spin tubes, shake flasks and bench scale bioreactors to compare cell culture performance parameters such as growth, productivity and product quality attributes in the form of isoform profiles and glycan antennarity structures. The results of the experiments showed that similar cell culture performance and product quality could be achieved in spin tubes when compared to shake flasks. Additionally, bioreactor titers could be reproduced in spin tubes at high shaking rates and low working volumes, but with differing product quality. Cultivation at lower shaking rates in spin tubes and shake flasks produced a glycoprotein with a product quality slightly comparable to that from bioreactors, but with titers being only two thirds. © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.*, 26: 653–663, 2010
Keywords: spin tube, D-optimal design, design expert, glycosylation, erythropoietin

Introduction

Chinese hamster ovary cells (CHO) are the workhorse in the production of biopharmaceutical products as they are widely used in research. Their most important characteristic is, however, their ability to produce highly complex glycoproteins that are similar to the native biomolecules in the human body. The demand for these recombinant proteins in fundamental research and clinical applications is continually increasing.[1,2]

One such glycoprotein is recombinant human erythropoietin (r-HuEPO), a hormone that acts on the bone marrow stem cells to stimulate erythropoiesis.[3] The human erythropoietin gene has been mapped on chromosome 7q11-q22. The glycoprotein consists of 165 amino acids that form a polypeptide chain with two disulfide chains at Cys7-Cys161 and Cys29-Cys33, the former being essential for the biological activity of the glycoprotein.[4] It contains three N-linked (at Asn24, Asn38, and Asn83) and one O-linked (at Ser126) glycosylation sites.[5] Its molecular mass is approximately

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36,000 g/mol, 40% of which are carbohydrates. The bonded carbohydrates are not essential for the biological activity, however, they extend the half-life of the molecule in the body and prolong its *in vivo* activity by preventing its premature removal from the blood circulation by the liver.[6] As a therapeutic it is used for the treatment of anemia resulting from chronic kidney disease, chemotherapy, etc. Therapeutic products based on r-HuEPO have a combined market value of $11 billion.[7]

Laboratory research and development work on suspended CHO cell growth optimization is mostly carried out in shake flask vessels.[8] However, their geometry and size is not favorable with respect to capacity considerations as only approximately 50 shake flasks with a 250 mL nominal volume can simultaneously be placed in a standard incubator unit. By using spin tubes with the nominal volume of 50 mL and a tubular geometry, the incubator capacity can be tripled. Additionally, the use of small scale equipment is less demanding in resources and usually easier to handle. Thus, as the use of spin tubes could more than triple the experimental throughput in optimizing cultivation conditions for CHO cells, the need emerged to investigate whether equivalent growth characteristics, productivity, and product quality can be achieved in spin tubes and in shake flasks. Previous research work on spin tube characteristics was carried out by De Jesus et al. (2004).[9]

It was also the objective of this research to investigate how different were growth characteristics, productivity and product quality in smaller systems such as spin tubes and shake flasks compared with bench scale bioreactors where better control of process conditions influencing product quality, for example, pH and dissolved oxygen control, can be applied.[10,11]

Also, another important aspect of our research work was the use of design of experiments (DoE) methods, since they are in the pharmaceutical industry highly favored by the Food and Drug Administration (FDA) in its Process Analytical Technology (PAT) initiative.[12] In the pharmaceutical industry and especially in bioprocess development, factorial and response surface methods for process analysis and optimization have seldom been used.[13] They were usually applied in the optimization of medium formulations to increase cell growth or production.[14–18] Some researchers used DoE methods to investigate the combined effects of medium composition and process parameters like pH, temperature, etc.[19–23]

DoE methods can be divided into three functional classes, namely screening, full factorial and response surface designs.[24] One of the more complex response surface methods, a computer generated D-optimal design was applied to optimize the cultivation conditions in spin tubes for a CHO cell line expressing recombinant human erythropoietin.

Materials and Methods

Cell line and inoculum cultures

A stable in-house CHO dihydrofolate reductase negative ($DHFR^-$) cell line expressing r-HuEPO was used. Throughout the research three vials from an in-house working cell bank (WCB) were used for inoculum preparation. The cell culture from the first vial was used for the primary experiments, the second vial was thawed for all replication experiments and the third, final, vial was used for validating the statistical model. As the WCB was derived from a single clone, the cultures originating from each of the three vials were not expected to exhibit a significant vial-to-vial variation in growth and productivity characteristics, therefore, culture origin was not used as a researched factor in the experimental design. In addition, as previous research work demonstrated genetic stability of the used cell line for a period of 3 months (data not shown), the inoculum cultures were maintained for no more than 90 days. Since the growth and productivity characteristics of the individual inoculum cultures were assumed to be equal throughout the experimental work, the inoculum culture age was also excluded as a factor from the experimental design.

Medium

An in-house, animal component free medium supplemented with recombinant protein and plant hydrolysates for cultivating CHO cells was used for all experimental work. Glutamine and glucose were the two essential substrates measured in the medium, and glutamate, lactate, and ammonium were the major cell culture waste products measured at the end of the batch processes. For each of the three inoculum cultures a fresh medium lot was used. For all three used medium lots the same lot of plant hydrolysates was used. We know from experience that the lot-to-lot variation of the medium is minimal, therefore, these variations are insignificant and do not influence the experimental results.

Spin tube and shake flask cultures

Sterile single use spin tubes (Techno Plastic Products AG, Switzerland) with dimensions 30 mm × 115 mm and a nominal volume of 50 mL were used in the experiments. Every spin tube screw cap has a hydrophobic gas-permeable membrane with a pore size of 0.22 μm attached to its bottom and five circular openings with different diameters (0.4, 0.6, 1.0, 1.5, and 2.0 mm) at the top to enable the transfer of gasses. During the experimental runs all five aeration vents were kept open.

As reference systems to which the spin tubes were compared, sterile 250 mL single use shake flasks at 50 mL working volume (Corning Incorporated Life Sciences) that are routinely used in cell culture development were employed. Shake flasks of various nominal volumes from the same manufacturer were also used for the maintenance of inoculum cultures.

All the small scale cultures were incubated in ISF1-X orbital shaker incubators (Kühner AG, Switzerland) without humidity control, and with the shaking diameter set to 50 mm, at 37°C and 10% CO_2. The inoculum and reference shake flask cultures were shaken at 90 rpm. The shaking rates and working volumes for the spin tube experiments were defined via the response surface design.

The seeding cell density for the spin tube, shake flask and the inoculum cultures was 2×10^5 viable cells/mL. The inoculum cultures were re-seeded into a fresh medium twice a week and were used on demand to start each new set of experimental runs. The duration of the experiments in the spin tubes and shake flasks was restricted to 7 days, since experience has shown that some of the substrates in the medium could be depleted in this period of time. A shake flask reference run was carried out parallel to every spin tube experimental run. The spin tube runs were carried out in five

parallel vessels, whereas the shake flask runs were carried out in three parallel vessels. The results were then averaged. On the seventh day of cultivation the final volume in the spin tubes and shake flasks was measured to establish the evaporation rate in the systems, taking into account the volume removed by sampling.

In the validation experiments the two bioreactors were seeded first. Approximately half an hour after seeding, part of the culture was taken from one of the bioreactors and used to inoculate the spin tubes and shake flasks to ensure equivalent starting numerical viable cell densities.

Bench scale bioreactor cultures

A Biostat® Bplus Twin (Sartorius Stedim Biotech, Germany) bench scale bioreactor system equipped with a 5 L (working volume) glass vessel with an approximately 2:1 height to diameter ratio ($H = 0.345$ m, $D = 0.16$ m) was used to perform a 7 day batch process during the validation experiments. The vessel did not contain baffles, was equipped with two marine impellers ($d = 0.07$ m) and aerated with a ring-sparger. The pH was maintained at 7.0, the dissolved oxygen (DO) level held constant at 50% of relative air saturation and controlled by sparging air, oxygen and nitrogen into the culture. The stirrer speed was set to 100 rpm, the seeding cell density was 2×10^5 viable cells/mL and the working temperature was controlled at 37°C. The inoculum for the bioreactor cultures was prepared by expanding the inoculum culture from 250 mL to 3 L (1000 mL working volume) shake flasks, incubated at the same conditions as those previously mentioned.

Viable cell density and cell viability measurements

An automated image analysis cell counter ViCell XR (Beckman-Coulter, Inc.) using the trypan blue exclusion method was used to determine the numerical cell density and viability in the samples. Sampling, 1 mL of cell suspension from each spin tube and shake flask, was performed on days 2, 5, and 7, and daily from the bioreactor runs.

Metabolite concentration measurements

Metabolite concentrations, using 1 mL samples from each spin tube and shake flask, were measured only on day 7 by an automated biochemical analyzer BioProfile 400 (Nova Biomedical). The metabolite concentrations in the bioreactors were measured daily via potentiometric electrodes for pH and partial pressure of carbon dioxide (pCO_2), ammonium, sodium, and potassium ions or amperometric electrodes for partial pressure of oxygen (pO_2), glucose, lactate, glutamine, and glutamate.

Product titer measurements

Product titers were measured by a reverse phase high performance liquid chromatography (RP-HPLC). The 1 mL samples from spin tubes, shake flasks, and bioreactors were taken on the seventh day of cultivation. They were first centrifuged for 5 minutes at 1000 g and 24°C, then filtered through a 0.2 μm sterile filter membrane and divided into two aliquots of 0.5 mL each. One was used for RP-HPLC analysis, and the other was saved as back up.

Determination of glycosylation patterns

The glycosylation patterns were determined only in the validation step, as higher culture volumes were needed to isolate a sufficient quantity of product for the glyco-analysis. Therefore, the number of spin tubes had to be modified: for the setting of 300 rpm shaking rate and 14 mL working volume, 15 spin tubes were used, and, for the setting of 180 rpm shaking rate and 30 mL working volume, 5 spin tubes were used. Additionally, three shake flasks at 90 rpm shaking rate and 50 mL working volume were used as reference. The sampling protocol for the validation experiments was the same as the one used before. Each individual vessel was first centrifuged for 5 minutes at 1000 g and 24°C. The remaining culture volumes were then pooled with respect to the above mentioned settings. The pooled volumes were then filtered. This pretreatment was necessary to remove cells and cell debris before further purification steps.

After this pretreatment the samples had to be purified and concentrated via immunoaffinity chromatography before product quality attributes could be assessed, as higher product purity and product concentrations are needed for the glyco-analysis.

The isoform profiles of the intact glycoprotein were analyzed by capillary zone electrophoresis (CZE), based on overall charge. To determine the glycan composition according to the degree of antennarity, the N-linked glycans were first released from the protein by enzymatic hydrolysis. The released N-glycans were then further enzymatically desialylated. The obtained asialo glycans were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Experimental design

Design Expert version 7.1.3 (Stat-Ease, Inc.) was used to build the response surface design and to perform the statistical analysis of the experimental results. A D-optimal design was used to determine the influence of operation parameters on product titer, cell growth, metabolite concentrations, water evaporation, and other calculated factors in the spin tube system. The D-optimal design type was used because it enables the imposing of different restrictions on the design space. It is a computer generated design and is mostly used when the experimental region is irregular, for example if the region is not a cube or a sphere.[25] In such cases standard designs like for example central composite, face-centered composite, Box-Behnken, etc., may not be the best choice. Irregular regions of interest, as is the case in this article, occur fairly often. The experimental settings of the design are generated via the D-optimality criterion which seeks to maximize the determinant of the information matrix of the design. This criterion results in minimizing the generalized variance of the parameter estimates based on a predefined model.[26] However, the drawback of using the D-optimal response surface design is that it requires a higher number of experimental runs than simple factorial designs, especially when the list of influential parameters is long. Consequently, the focus has to be on a few of the most influencing parameters. From our list of candidate parameters composed of cultivation temperature, CO_2 level, orbital shaking diameter, seeding cell density, shaking rate and working volume, the last two were predicted to be the most influential ones due to the differences in the geometry of spin tubes and shake

flasks. It was, therefore, decided to focus the investigation on the influence of shaking rate (n) and working volume (V).

In generating a D-optimal design the user has to predict the form of the final statistical models, for example linear, two factor interaction (2FI), quadratic or cubic. If the form were too simple, this would result in the statistical models making a poor description of the system. On the other hand, if it were too complex, some experimental runs would become redundant. In this article it is assumed that a quadratic model would give a decent approximation of the actual response surface based on a reasonable number of experimental runs.

Although the shaking rates for the incubator units in principal could be set at a range from 0 to 300 rpm and the working volumes in the spin tube from 0 to 50 mL, the rate was restricted to an interval from 180 to 300 rpm, and the working volume from 14 to 34 mL. The lower limit for the volume was set so that minimal sampling was still possible, while the upper limit was set to 34 mL. On the basis of the previous research, the lower bound of 180 rpm on the shaking rate was decided to prevent sedimentation of cells. In addition, a linear constraint of shaking rate and working volume, was imposed for settings where the spin tube screw cap with an in-built gas-permeable membrane could be moistened due to culture splashing.

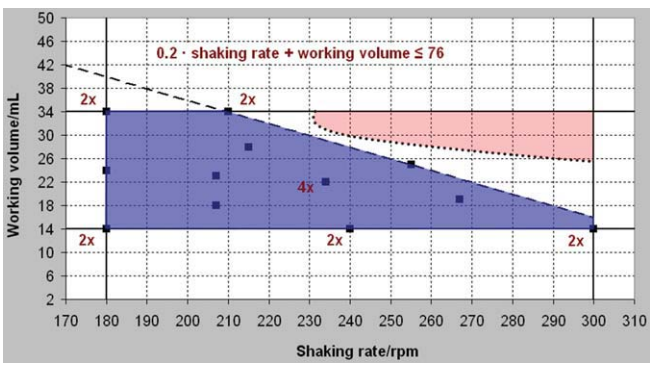

Figure 1. A graphical representation of the design space (dark shaded area).

Also presented are the non-linear restriction (dotted line), linearized non-linear constraint (dashed line), unfeasible region (light shaded area), working volume (V) and shaking rate (n) bounds (bold solid lines) and design points (■).

It should be noted that the restriction regarding screw cap membrane moistening was experimentally found as being non-linear (dotted line on Figure 1). Since the software allows only linear constraints to be inserted, the slope of the linear constraint was adjusted so that high working volumes were retained in the design space (linear constraint: $0.2 \times$ shaking rate $+$ working volume ≤ 76), but a part of the space around the smaller working volumes and the higher shaking rates was therefore lost. Since the design space is restricted to feasible operating conditions, the experimental points generated are also feasible. In addition, if the design space were unrestricted, and therefore bigger than when restricted, the design points would be generated far apart from each other and this would statistically weaken the resulting models.

In the generated design the central point at 234 rpm and 22 mL was replicated four times (Figure 1) to carry out an adequate curvature assessment. Five extreme points (Figure 1) in the design layout were also replicated to allow a good estimation of pure error.

It should be noted that a trade-off between the number of experimental runs, and the precision of the statistical model is frequently encountered. A larger number of time-consuming and expensive experiments have to be carried out to achieve higher model precision. If research costs are the limiting factor, fewer experiments can be carried out resulting in a weaker statistical model. As the duration of a single run in this research work was a whole week and the equipment capacity limited, it was not possible to perform an excessive experimental plan.

Results and Discussion

Experimental results

After the bounds for the shaking rate and working volume, as well as the linear constraint, were inserted into the software, a list of different factor settings was generated by the program. Table 1 shows the semi-random order for the

Table 1. Semi-random Run Order and the Corresponding Product Titers

Run Order	Point Type	Shaking Rate (rpm)	Working Volume (mL)	[a]Observed Titer (mg/L)	Calculated Titer (mg/L)	
1	Vertex	180	14	47	49	**Primary Runs**
2	Center of Edge	180	24	38	43	
3	Vertex	180	34	37	38	
4	Center of Edge	240	14	45	51	
5	Vertex	300	14	50	53	
6	Interior	215	28	34	32	
7	Center	234	22	35	35	
8	Vertex	210	34	30	29	
9	Center of Edge	255	25	31	29	
10	Interior	267	19	43	37	
11	Axial check point	207	18	39	44	
12	Interior	207	23	33	38	
13	Vertex	180	14	59	49	**Replicated Runs**
14	Vertex	180	34	47	38	
15	Center of Edge	240	14	71	51	
16	Center	234	22	35	35	
17	Vertex	300	14	49	53	
18	Center	234	22	35	35	
19	Vertex	210	34	26	29	
20	Center	234	22	28	35	

[a] Average from five parallel spin tube values.

Table 2. Statistical Summary Derived from the Software Output

Response	Transformation	Model	Adjusted R^2	Predicted R^2
[a]Evaporation/mL	Power	RLinear	0.2808	0.1191
[a]Maximal C_{viable}/(10^6 cells/mL)	None	RLinear	0.2435	0.0799
[b]Viability/%	None	Linear	0.2278	0.1100
[b]Titer/(mg/L)	Inverse	2FI	0.6911	0.5945
[c]Specific growth rate/day^{-1}	None	Mean	/	/
[d]Specific productivity/(pcd)	Power	2FI	0.5521	0.3678
[b]pH/1	None	Mean	/	/
[b]pO_2/(mm Hg)	None	Mean	/	/
[b]pCO_2/(mm Hg)	None	Mean	/	/
[b]Glutamine/(mmol/L)	None	Mean	/	/
[b]Glutamate/(mmol/L)	None	Mean	/	/
[b]Glucose/(g/L)	None	Mean	/	/
[b]Lactate/(g/L)	None	Mean	/	/
[b]Ammonium/(mmol/L)	None	RLinear	0.1671	0.0028
[b]Na^+/(mmol/L)	None	RLinear	0.2928	0.1345
[b]K^+/(mmol/L)	None	Linear	0.6677	0.6060
[b]Osmolality/(mOsm/kg)	None	RLinear	0.3129	0.1194
[b]Air Saturation/%	None	Mean	/	/
[b]CO_2 Saturation/%	None	Mean	/	/
[b]HCO_3^-/(mmol/L)	None	2FI	0.3592	0.1990

[a] Throughout of a 7 day cultivation;
[b] Measured at the end of the cultivation;
[c] Calculated for the exponential growth faze based on viable cell density, from day 2 to day 5;
[d] Titer on day 7, divided by the area under the viable cell growth curve, approximated with the trapeze method.
C_{viable} – Numerical viable cell density;
pO_2 – Partial pressure of oxygen;
pCO_2 – Partial pressure of carbon dioxide;
RLinear – Reduced linear statistical model;
2FI – Statistical model with a two factor interaction and linear terms;
Adjusted R^2 – Adjusted coefficient of determination;
Predicted R^2 – Predicted coefficient of determination.

minimal number of generated experimental runs observed. Software calculated titers are also presented.

As research time and equipment capacity were limited, all replicates (runs 13–20) were carried out after the original distinctive runs were finished. Replicates should normally be executed between distinctive runs to prevent the results being compromised by other potential influential factors, for example, medium age, lot-to-lot variations, etc. For example, if the characteristics of the culture deteriorated with time, the results of the original experimental runs could not be compared to the ones obtained for replicates, if the latter were performed much later.

After executing all the runs in the aforementioned order, the measured values from all 20 experimental runs were inserted into Design Expert and analyzed. Altogether, the data for 20 responses (titer, metabolite concentrations, etc.) were inserted. The analysis of adjusted and predicted coefficients of determination (R^2-values) which should be higher than 0.6 indicates that the majority of responses cannot be described with the chosen statistical models. The software uses the adjusted R^2 as an indicator of how well the model fits the experimental points, and the predicted R^2 to indicate how well the model can predict future experimental runs. A summary of the results from the analysis of variance (ANOVA) is presented in Table 2.

Table 2 shows that only the models for the product titer (p) and the concentration of potassium ions have adequately high adjusted and predicted R^2-values. Moreover, in the sense of titer they differ less than 0.2 from each other. The other values for the adjusted and the predicted R^2 are lower than 0.6 which is why the other responses are ignored in the optimization step. Since the concentration of potassium ions is not regarded as a major response, its model was also excluded from further consideration. For some responses, for example, pH, glucose concentration, etc., only the mean value of all experimental runs could be used to describe the system, which leads to the conclusion that specified shaking rates and working volumes seem to have no significant effect on these responses in the explored design space. Consequently, only the statistical model for the titer was used in the continuation of the research. The detailed ANOVA results for the product titer model are presented in Table 3.

The α-value of statistical significance (Table 3) was set to 0.100, which means that all factors whose P-values was higher than 0.100 were excluded from the statistical model, as they were non-significant. The ANOVA results show that the proposed model is significant, and that the shaking rate and the working volume have a certain influence on product titer in the specified design space. The lack of fit value is beyond the 0.050–0.100 region, which suggests that the model can be used as a prediction tool. Note that the prediction error sum of squares (PRESS) value–an indicator for the prediction quality of the model is very low (0.0003), and the value of the statistical parameter adequate precision (signal to noise ratio) is larger than 4 (10.290), which means that the background noise (variation) is lower than the measured response values. The overall analysis thus showed that the statistical model that describes the product titer value on the seventh day of cultivation is significant and can be used for modeling and optimization purposes in the defined design space.

Two different representations of the statistical model for product titer are shown in Table 3. In the first equation all the factors are coded and, therefore, the coefficients before the factor can be directly compared to each other. For

Table 3. ANOVA for the Product Titer Model

Response:			Titer on Day 7		
Transformation:			Inverse		
Backward Elimination Regression with $\alpha = 0.100$					
ANOVA for Response Surface 2FI Model					
Source	Sum of Squares	df	Mean Square	*F*-Value	*P*-value
Model	555.52	3	185.17	15.17	< 0.0001
A: Shaking rate	192.12	1	192.12	15.74	0.0011
B: Working volume	526.39	1	526.39	43.13	< 0.0001
A · B: Interaction	197.26	1	197.26	16.16	0.0010
Residual	195.28	16	12.21		
Lack of Fit	84.77	8	10.60	0.77	0.6416
Pure error	110.51	8	13.81		
Corrected Total	750.80	19			
Standard Deviation	0.004		R^2	0.7399	
Mean	0.026		Adjusted R^2	0.6911	
Coefficient of Variance/%	13.34		Predicted R^2	0.5945	
PRESS	0.0003		Adequate Precision	10.290	

Final Equation in Terms of Coded Factors:
$$\text{Titer} = 1/(0.0320 + 0.0082 \cdot A + 0.012 \cdot B + 0.0090 \cdot A \cdot B)$$

Final Equation in Terms of Actual Factors:
$$p = 1/(0.056806 - 0.000223 \cdot n - 0.002421 \cdot V + 0.000015 \cdot n \cdot V)$$

α – Level of statistical significance;
2FI — Statistical model with a two factor interaction and linear terms;
PRESS – Prediction error sum of squares;
df — Degrees of freedom;
R^2 – Coefficient of determination;
Adjusted R^2 – Adjusted coefficient of determination;
Predicted R^2 – Predicted coefficient of determination;
Adequate precision – Signal to noise ratio;
F-value – Calculated value from a hypothesis test;
P-value – Probability level;
p – Product titer, mg/L;
n – Shaking rate, rpm;
V – Working volume, mL.

example, the factor working volume (B) has a more powerful influence on the product titer than the factor shaking rate (A) or their interaction (A · B). The second equation is not coded, which makes a direct comparison between the influences of both factors not possible.

In addition to the ANOVA analysis a graphical diagnostic analysis of the statistical adequacy of the model was also performed. Figure 2 summarizes the most important diagnostic graphs for the product titer model. As can be seen in Figure 2A, all the residuals (points) follow a straight line, that is, they are normally distributed. If the points would be distributed in an "S" pattern around the straight line, the residuals would be non-normally distributed. The data in Figure 2B confirm the presumption of constant variance, since no relevant pattern (i.e., "megaphone shape; <") is visible. Figure 2C shows the predicted vs. actual responses plot where the values should be randomly scattered around the 45° line, as is the case here. Grouping on one or the other side of the line is not desired, as that could indicate over or under prediction by the model. The Box-Cox plot (Figure 2D) suggests an inverse transformation (e.g., $1/p$), to generate a statistically relevant model. Transforming data minimizes the effect of extreme values (outlier) on the model. The externally Studentized residuals plot (Figure 2E) shows that no value exceeds the limit value (red horizontal lines), therefore, there are no outlier values in our measurements. Outliers can deform the statistical model and make it thus less precise. Finally, the Cook's distance plot (Figure 2F) demonstrates that run 14, that is, the highest point, has a stronger influence on the statistical model than the other design points, but as it is not an outlier this is not a cause for concern. The graphical analysis thus confirmed that the statistical model should be able to predict the product titer in the restricted design space.

The main objective here was to define the operational settings in which spin tubes have approximately the same titer as the shake flasks at standard conditions (90 rpm, 10% CO_2 and 37°C). For this purpose several experiments in shake flasks were preformed to define a reference titer. The average titer from all reference runs was 40 mg/L. This titer can be achieved at a broad range of settings in spin tubes (Figure 3A, contour line for titer 40 mg/L). As it is not practical to use several possible cultivation settings, a relevant point setting had to be defined and the software in-build optimizer was thus used.

The response surface of the final titer can also be drawn as a 3D grid plain (Figure 3B), and consequently be used as a graphical representation of the fit test. It also shows that the response surface is curved.

Alongside the equality setting, this research also wished to determine those settings in which maximal product titer could be obtained. Figure 3A shows that the maximal product titer (the right hand corner) can be achieved at settings of low working volumes and high shaking rates. We assume that material transfer (metabolites, gasses) is better at these settings than in larger working volumes and lower shaking rates, which consequently could influence productivity and also product quality. After the model was defined, it could be used to optimize the operational settings.

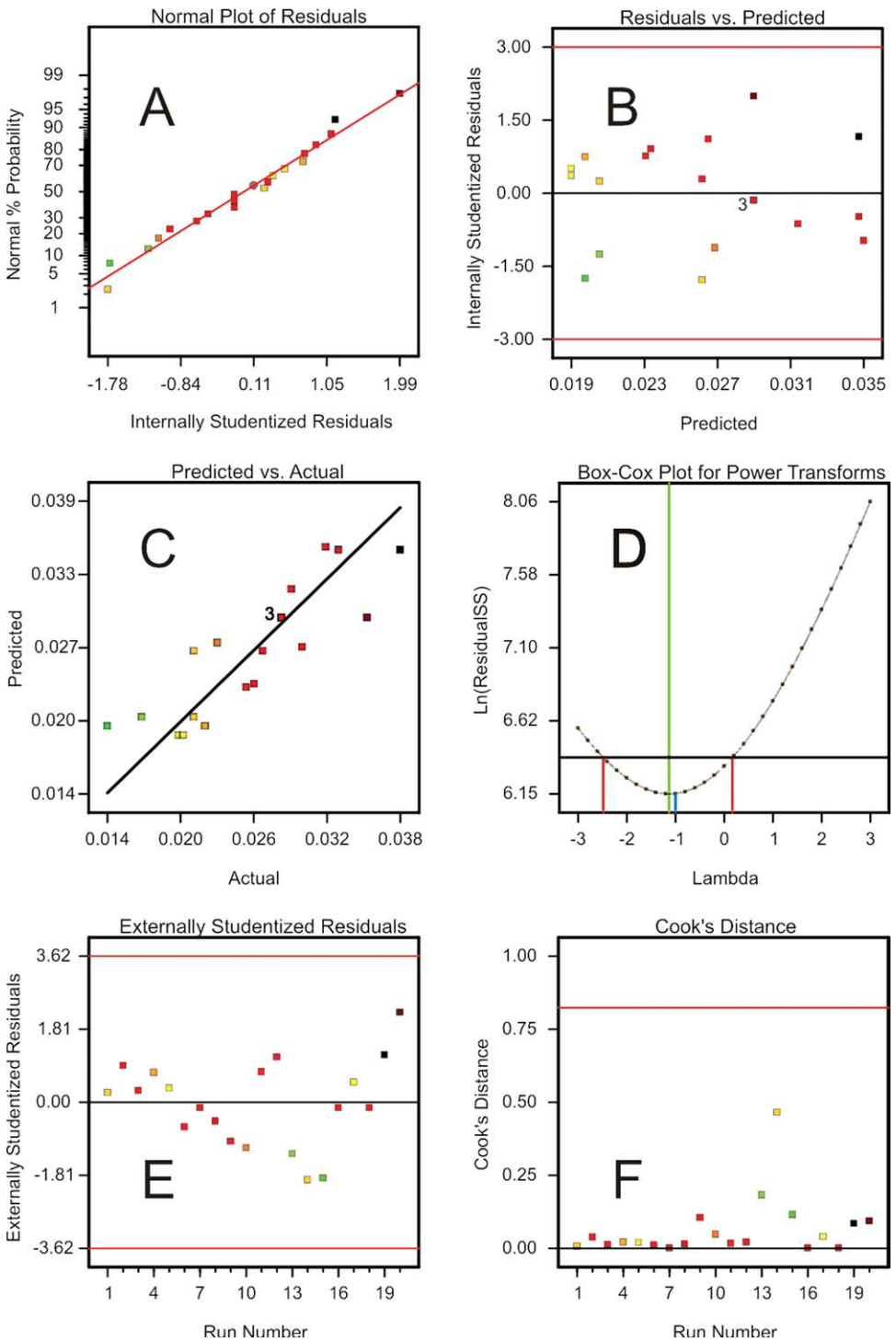

Figure 2. Graphical diagnostic analysis.

(A) Normal plot of residuals. Residuals (points) are calculated as differences between actual and predicted response values for each point. Internally Studentized residuals are residuals divided by their estimated standard deviations. Darker colored points in all Figures represent lower titers. (B) Residuals vs. predicted responses plot. The software presents the predicted and actual responses in coded format. All response values are in the three sigma limits (red horizontal lines). (C) Predicted vs. actual responses plot. Actual and predicted response values are equal if they fall onto the 45° line. (D) Box-Cox plot. The used optimal Lambda value (blue vertical line) represents the power number of the transformation (e.g., $1/p$ or Lambda $= -1$). The red vertical lines represent the 95% confidence interval for the theoretical optimal Lambda value (green vertical line). (E) Externally Studentized residuals plot. Externally Studentized residuals are calculated as internally Studentized residuals where the run in question is left out from the analysis. The run number represents the sequence of executing the experiments. (F) Cook's distance plot. A larger Cook's distance represents a higher effect of the data point on the model.

Optimization

The objective was to determine two optimal configurations: (i) the first one for cases where the product titers in a seven day batch were equal in the spin tubes and the shake flasks–point of equality, (ii) and the second one for cases where a maximal titer could be achieved in the spin tubes–point of maximal titer. An additional objective in the first case was the simultaneous maximization of the working volume, because of sampling and amount of product produced. The optimal settings obtained by Design Expert are the following:

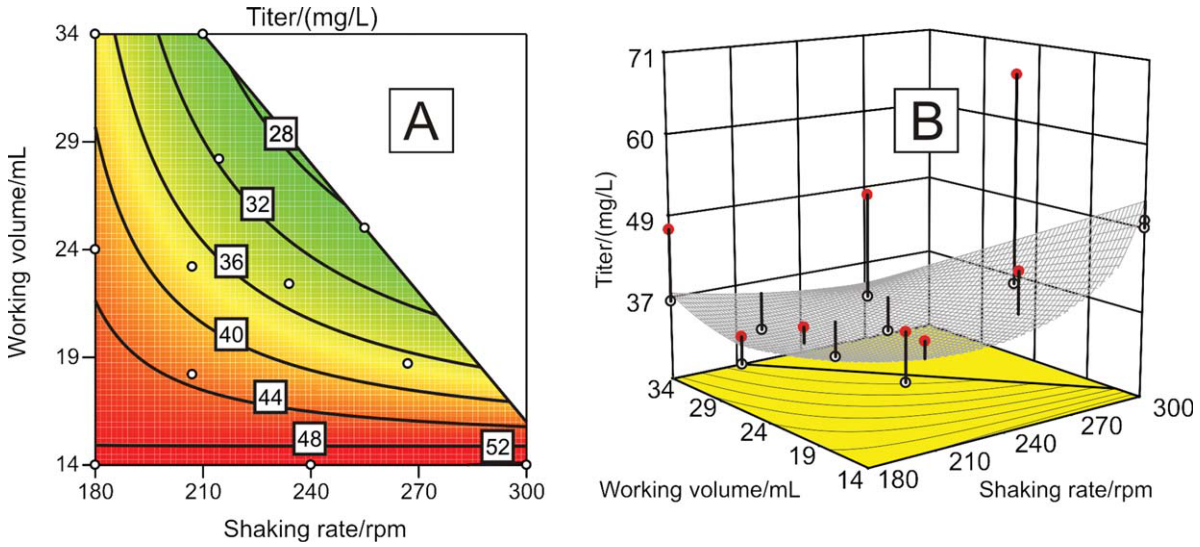

Figure 3. Graphical representation of the mathematical model.

(A) Contour plot for the product titer on the seventh day of cultivation. Titers calculated from the model are presented in brackets. The unfeasible area is presented as white space. Contour lines link areas with equal titer values. (B) 3D grid representation of the final titer. Experimental results above the response surface (●) and experimental results below the response surface (○) are shown. The variance in values of replicated points can also be seen. The curvature of the response surface is noticeable.

Table 4. Validation for Calculated 95% Prediction Intervals (PI) for Product Titer

				Average Titer/(mg/L)			
	Setting	Shaking Rate (rpm)	Working Volume (mL)	Low PI	Predicted	High PI	Observed
Spin Tube	A	180	30	30	40	60	54
Shake Flask	B	90	50	/	/	/	50
Spin Tube	C	300	14	36	53	97	81
Bioreactor	D	/	5000	/	/	/	78

Shaking rate of 180 rpm and working volume of 30 mL for the point of equality;

Shaking rate of 300 rpm and working volume of 14 mL for the point of the maximal titer.

Design Expert was able to predict the values of the product titers at both settings accompanied by the intervals of prediction (PI) for the calculated values. The results from the validation run should fall into these intervals if the statistical model is correct.

Model validation and system comparison

The settings determined in the optimization step were used to validate the statistical model. The predicted and experimental results for the product titer are compared in Table 4.

Table 4 shows that the spin tube system at the point of equality (setting A) and the shake flask system at setting B have almost equal product titers. Also, the spin tube setting at high shaking rates (C) produces a higher product titer than settings A and B, as predicted by the model. All the results fall into the software calculated 95% prediction intervals. However, the value for the product titer at setting C is much higher than the predicted value of 53 mg/L, but comparable to the value produced in the bioreactor system run at standard operating settings. The other two titer values for settings A and B are quite near the calculated value of 40 mg/L. Based on these results it could be concluded that the statistical model for product titer prediction in spin tubes is valid for the specified design space.

The validation run was additionally used to compare product quality attributes and viable cell growth curves between systems of different scales. The goal was to examine if the point of equality for the titer can be extrapolated also to the chosen product quality attributes.

Isoform distribution (Figure 4A) representing the charge distribution of the intact glycoprotein and glycan group distribution according to the degree of glycan group antennarity (Figure 4B) were determined as two possible quality attributes, where higher ranked isoforms and tetra- and triantennary groups are favored. Based on scientific knowledge of the biological system and the analytical error of the methods used, a 10% difference in values was chosen as the criterion for a significant discrepancy between the product quality attributes.

Figure 4 shows evidence confirming the possibility that the product quality in the spin tubes and that in the shake flasks (settings A and B) are equal. Slightly larger differences were observed only at the values of isoform 8 (Figure 4A) and the biantennary glycan structures (Figure 4B). Both Figures were normalized, setting C being chosen as the normalization standard.

Figure 5 shows a graphical presentation of the three classes of glycans based on their antennarity, mentioned in Figure 4B.

Higher ranking isoforms are desired as the lack of terminal sialic acids from carbohydrate chains decreases the *in vivo* activity of the glycoprotein.[29] The results of the isoform distribution (Figure 4A) show that at setting C (spin tube at the point of maximal titer) more lower ranking isoforms are produced than in other settings, while the distribution in the bioreactor setting (D) is essentially equal to those in settings A and B, a slight difference occurring only at the values for

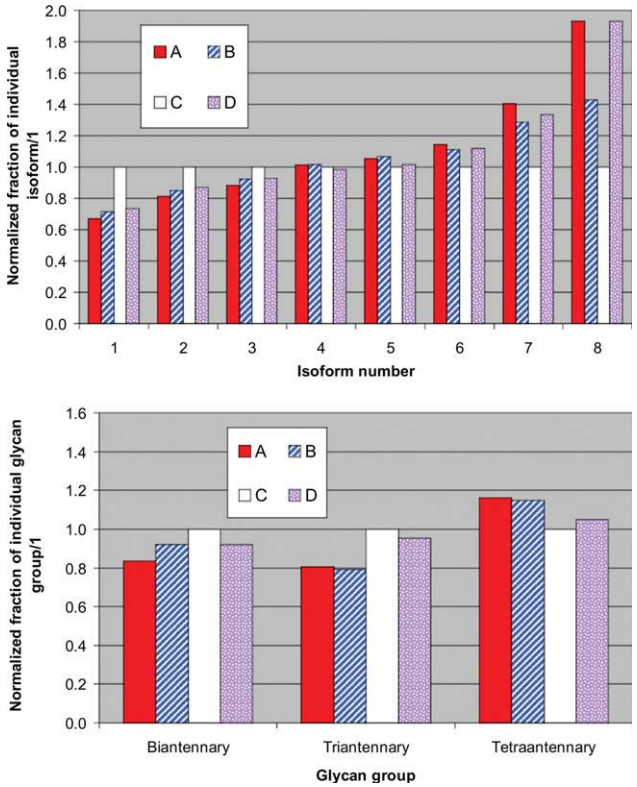

Figure 4. Comparison of quality attributes.

(A) Relative proportions of product isoforms for the settings (A, B, C and D) discussed in Table 4. The isoforms are ranked from 1 to 8, where isoform 1 has the smallest charge and is thus the least sialylated. On the other hand, isoform 8 has the highest charge and is thus the most sialylated. (B) Relative proportions of product glycan groups for the settings (A, B, C and D) discussed in Table 4. Three main glycan classes differentiated through antennarity are presented.

isoform 8. This means that if only the isoform profile were measured, the bioreactor, the spin tube and the shake flask systems would have equal performance, differing only in product titer values.

It is interesting to note that a significant difference in triantennary and tetraantennary structures can be observed between the bioreactor on the one side and the spin tube and shake flask systems one the other side (Figure 4B). Multi-branched structures are much desired as these structures may function as an intramolecular drug-delivery system. They also have a higher number of bonding sites for sialic acid molecules that are terminally bonded to the carbohydrate chains than less-branched structures. In such cases, an antennary distribution that shifts toward tetraantennary structures is desired. The proportion of higher antennary structures in bioreactors is lower than in spin tubes and shake flasks at settings A and B, but their isoform distribution is quite similar. It is possible that, although the glycan structures produced in bioreactors are less branched, the sialylation is better than in spin tubes and shake flasks at the used settings. In the spin tube and shake flask systems the glycoprotein could have more branched glycans chains, but they are not all sialylated. Based on these observations, it can be concluded that the distribution of glycan structures for the bioreactor is not comparable to those of settings A and B, while it is comparable to the one of the spin tube at the point of maximal titer (C). The distribution of glycan structures for setting A and B is also comparable, a slight discrepancy occurring at the fractions of the biantennary structures.

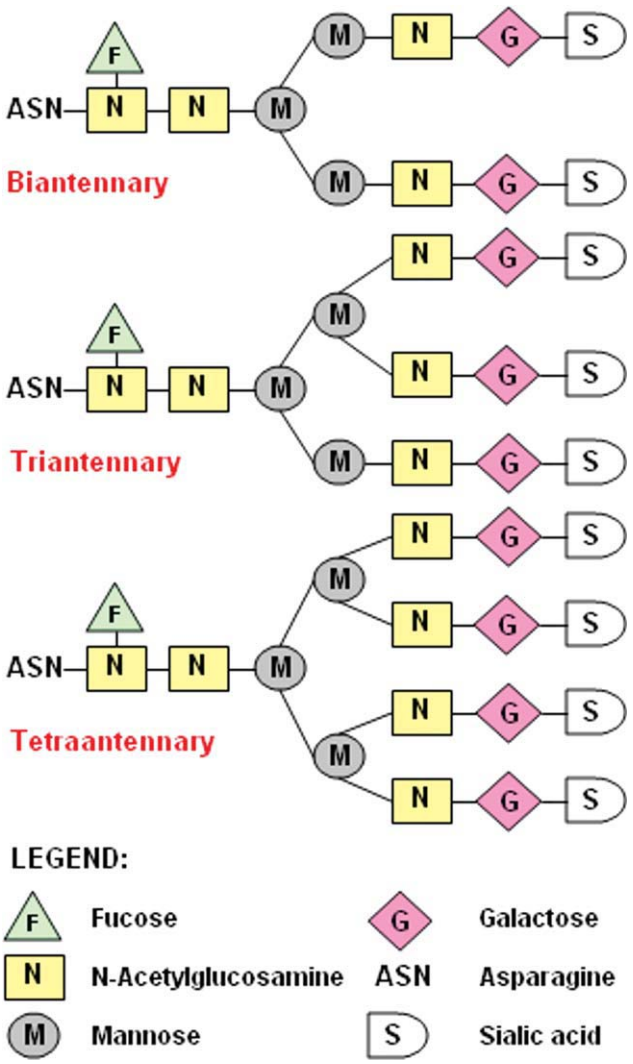

Figure 5. A schematic presentation of basic gycan structures.

For triantennary and tetraantennary structures more conformations exist, but not all are presented here. Detailed graphical presentations of all the different antennary structures were published by Takeuchi M. and Kobata A., also by Sasaki H. et al.[27,28]

In addition, the viable cell growth curves were also compared across the systems investigated in the validation run. The growth curves for the four settings in Table 4 are presented in Figure 6.

It should be noted that the growth in the bioreactors was slower and the maximal viable cell density lower compared to the other two cultivation systems, which may explain the differences in the glycosylation patterns. The values in Table 4 and the growth curves in Figure 6 suggest that higher titers are achieved in spin tube and shake flask systems when cell growth, too, is higher. Bioreactor measurements however do not seem to share this conclusion. The less pronounced cell growth in bioreactors could be explained with the use of standard, possible non-optimal settings.

Based on these observations, it can be concluded that spin tubes at the point of equality settings (180 rpm, 30 mL working volume) and shake flasks at standard settings (90 rpm, 10% CO_2 and 37°C) perform equally as regards product titer, the isoform profile and the degree of glycan chain antennarity. A slight discrepancy, however, occurs in the

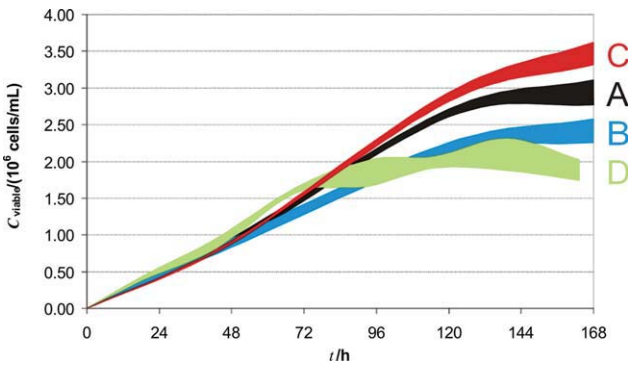

Figure 6. Comparison of growth curves.

Viable cell density growth curves for the settings (A, B, C, and D) as discussed in Table 4. 95% confidence intervals are presented. At $t = 120$ h the curves A, B, and C enter into the stationary growth phase, the death phase was not measured as the experiments were terminated at $t = 168$ h. Experiment D was terminated slightly before the other ones. The growth curve at setting D enters the stationary growth phase much earlier then the others. The seeding densities at the beginning of the batch processes were identical.

percentage of biantennary structures and in the proportion of isoform 8.

On the other hand, cell growth and product quality from the spin tube and shake flask systems differ from those in the bioreactor system, due to considerably different cultivation conditions. It is known that product quality is dependent on the environmental parameters such as pH value, osmolality, metabolite concentrations, etc. In our experiment, especially the pH profile and the gassing regime of the spin tube and shake flask systems were significantly different when compared to the bioreactor system, where both parameters are controlled. From our measurements it is clear that the pH value in spin tubes and shake flasks (settings A, B, and C) is considerably lower at the end of the batch cultivation, that is, at approximately 6.7, compared with the bioreactor set point of 7.0 (setting D). Osmolality on the other hand was quite similar for all three systems, a higher value was achieved at the spin tube setting of 300 rpm and 14 mL working volume. It is, however, difficult to asses the effect of elevated osmolality as it was not measured directly, but calculated by the BioProfile analyzer from the solute concentrations, therefore, uncertainties do exist. Higher values of partial carbon dioxide pressure were noticed in spin tubes and shake flasks compared to bioreactors, and also lower ammonium levels were noticed in the bioreactors. Lower pH values in the small scale systems can be linked to elevated carbon dioxide levels. In all three systems the substrate glutamine was nearly depleted and may have been the growth limiting factor in the experiments. The glucose concentrations at the end of the batch culture were twice as high in spin tubes and shake flasks as in the bioreactors, therefore, the glucose concentrations should not have been growth limiting. However, as the observed cultivation systems are multiparameter dependent environments, it is very difficult to pinpoint the influence of a single or a few parameters on cell growth and product quality.

Conclusion

A response surface D-optimal design was used to optimize the cultivation conditions in spin tubes for a CHO cell line producing erythropoietin. Two distinct settings were determined, namely one where the product titer was equal in the spin tubes and the shake flasks, and one where a maximal titer in the spin tubes could be produced. The statistical model for the product titer on the seventh day of cultivation, with shaking rate and working volume as influencing factors, was verified and can be used as a prediction tool.

Product glycosylation patterns were found to be equal for validated settings in spin tubes and shake flasks, but are not fully comparable to the glycosylation of the product from the bioreactors at the chosen similar settings. Bioreactor systems are more controllable, but also more complex than the smaller systems. Therefore productivity and product quality attributes can be influenced by many factors that are present in bioreactors, but not present in small scale systems such as spin tubes and shake flasks, or vice versa.

It can be concluded that the objectives of this research were fulfilled, as settings of the shake rate and working volume were found where the spin tube system is comparable to the shake flask system. Spin tubes can therefore be used to increase the experimental throughput, the only limitation being the culture volume needed to isolate sufficient material for extensive product quality analysis (e.g., glycans). Although these conclusions are valid only for the cell clone used in this research and further work has to be done to asses the influence of the chosen factors on different clones, the methodology used and presented in this article is generally applicable.

Acknowledgments

The authors gratefully acknowledge the partial financial support in the grant No. 3211-06-000501 of the Ministry for Science, Technology and Development of the Republic Slovenia. The authors would also like to thank Lek Pharmaceuticals d.d., a Sandoz Company for its support, especially the Biopharmaceuticals departments of Bioprocess Development and Analytics.

Notation

Adequate precision = signal to noise ratio
Adjusted R^2 = adjusted coefficient of determination
ANOVA = analysis of variance
Asn = asparagine, an amino acid
C_{viable} = numerical viable cell density
CHO = chinese hamster ovary cells
CZE = capillary zone electrophoresis
Cys = cysteine, an amino acid
D = bioreactor diameter
d = impeller diameter
df = degrees of freedom
$DHFR^-$ = dihydrofolate reductase negative cell line
DO = dissolved oxygen
DoE = design of experiments
H = bioreactor height
F-value = calculated value from a hypothesis test
FDA = food and drug administration
HPAEC-PAD = high performance anion exchange chromatography with amperometric detection
n = shaking rate
p = product titer
PAT = process analytical technology
pCO_2 = partial pressure of carbon dioxide
PI = prediction interval
pO_2 = partial pressure of oxygen
Predicted R^2 = predicted coefficient of determination
PRESS = prediction error sum of squares

P-value = probability level
R^2 = coefficient of determination
r-HuEPO = recombinant human erythropoietin
RLinear = reduced linear statistical model
RP-HPLC = reverse phase high performance liquid chromatography
Ser = serine, an amino acid
t = time
V = working volume
WCB = working cell bank
2FI = statistical model with a two factor interaction and linear terms
α = level of statistical significance

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Manuscript received Jun. 19, 2009, and revision received Oct. 24, 2009.