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Microplates with integrated oxygen sensing for medium optimization in animal cell culture

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Abstract

A new approach using microtiter plate cultivation with on-line measurement of dissolved oxygen (DO) was applied for medium optimization of mammalian cell culture. Applying dynamic liquid phase balance, oxygen uptake rates were calculated from the DO level and used as an indicator for culture viability. The developed method was successfully applied to optimization of the concentration of glucose, glutamine and inorganic salts for cultivation of a Chinese Hamster Ovary (CHO) cell line. Using 2^3 full factorial central composite design, the optimum medium composition could be identified in one single run. The concentration of inorganic salts had a significant influence on cultivation. The developed method exhibits high potential to improve procedures of medium optimization for animal cell cultivation by allowing the investigation of large sets of potentially important variables in short time and with reduced effort.

Introduction

Development of processes involving mammalian cell cultures producing therapeutically important proteins is generally very time consuming. Medium optimization plays a key role in this process (Hesse and Wagner 2000). Classically this is done by the addition or deletion of components, one at a time, to see their influence on the process. However, this approach has a lot of problems associated with it including false optimums, much experimentation, no information of interactions etc. Therefore, statistical design is used to minimize experimental efforts and provide relevant information (Ertola et al. 1995; Massart et al. 1997). Essential items to start such optimization are the selection of potentially influencing parameters, the measurement of the output and the method of experimental design applied. The selection of input variables depends strongly on the specific problem. The inputs could involve specific individual components of a medium or complex media supplements or operating variables such as pH or temperature. The output is generally the growth rate or production rate (Castro et al. 1992; Lee et al. 1999). However, for the primary phases this is generally the cell proliferation. Recently Chun et al. (2003) applied statistical experimental design to identify growth factors in an overall effort to accelerate recombinant CHO medium development with cell proliferation as the output variable. In this context the measurement of oxygen uptake rate (OUR) as the output parameter is of high interest because it is a known indicator for metabolic activity and directly reflects culture viability (Ramirez and Mutharasan 1990; Eyer et al. 1995; Schoenherr et al. 2000). Despite the use of experimental design strategies (Palmqvist et al. 1999; Ellaiah and Adinarayana 2002) the number of experiments required for optimization studies is still large

considering the large number of potential influencing parameters in mammalian cell culture. Therefore, high-throughput methods for cultivation and on-line monitoring such as 96 well microtiter plates with on-line oxygen sensing are needed. The application of these plates for culture viability measurements using oxygen uptake rate has been shown (Deshpande and Heinzle 2004). The viability-OUR relationship changes when the specific OUR is changing. One can, however, expect that in almost any case less favorable medium composition would decrease both culture viability as well as cell specific OUR. This additive effect would even simplify finding an optimum medium composition. Here we show the applicability of these microplates for medium optimization using statistical experimental design with oxygen uptake rate as a parameter.

Materials and methods

Oxygen-sensor microtiter plate

The oxygen-sensor microtiter plates (OXOPLATE OP96F) were provided by PRESENS, Precision Sensing GmbH (Regensburg, Germany). They were packed sterile, manufactured from polystyrene in the common 96 round well format and supplied with a lid. An oxygen sensor is immobilized on the round bottom of each well. This sensor can be read from the bottom side with commercially available readers. The sensor contains two different dyes. One is the oxygen indicator. Its fluorescence intensity I_{ind} is dependent on the concentration of oxygen in the sample filled into the well. The other dye is the reference. Its fluorescence intensity I_{ref} is independent of the oxygen concentration. Using the luminescence intensities, the ratio I_R can be calculated. This referenced signal I_R correlates with the concentration of oxygen.

$$I_R = \frac{I_{ind}}{I_{ref}} \tag{1}$$

Calibration is done by the determination of the zero point by the chemical removal of dissolved oxygen using dithionite and by subsequent saturation with oxygen from air. The relationship between fluorescence intensity and dissolved oxygen concentration is nonlinear and described by the Stern–Volmer equation for collision quenching:

$$\frac{I_R}{I_{R,0}} = \frac{1}{1 + K_{SV}[O_2]} \tag{2}$$

Where $I_{R,0}$ is the fluorescence intensity ratio in the absence of oxygen, I_R is the fluorescence intensity ratio at the oxygen concentration $[O_2]$ and K_{SV} is the Stern–Volmer constant (John et al. 2003).

Measurements were carried out in a fluorescence reader, equipped with integrated shaker and temperature control (Fluoroskan Ascent, Labsystems, Vantaa, Finland) using dual kinetic mode. The fluorescence intensities were measured with the filter combinations 544 nm/650 nm and 544 nm/590 nm. The first combination measured fluorescence depending on oxygen concentration, whereas the latter showed the oxygen independent constant fluorescence of the reference signal.

Cell line and medium

The Chinese Hamster Ovary (CHO) cell line T-CHO ATIII, obtained from Gesellschaft for Biotechnologische Forschung mbH (Braunschweig, Germany), was used for this research. The cells produce recombinant antithrombin III, which has clinical applications for its coagulation inhibitory activity. The cells were adapted to grow in a protein and peptide free, chemically defined medium (SMIF 6, Life Technologies, Karlsruhe, Germany). The cells were grown in 250 ml spinner flasks placed in a 12% CO_2 humidified gas controlled incubator at 37 °C. The cultures were maintained in growth phase by changing the medium using the serial dilution method (Morris et al. 1997).

Microplate culture of cells in the fluorescence reader was carried out at 37 °C by employing a shaking frequency of 660 min^{-1} with a shaking diameter of 2 mm.

Statistical experimental design

The present experiments are aimed to show the applicability of microplates with immobilized oxygen sensors as a system to study medium optimization in primary stages. The medium components varied were inorganic salt mixture

(A), glutamine (B) and glucose (C). The 2^3 full factorial central composite design used in response surface methodologies was applied. This consists of the evaluation of the relationship between controlled experimental factors and the measured response, in this case being the oxygen uptake rate of the cells at the end of the cultivation time. For statistical calculations, the variables were coded as according to the following equation.

$$x_i = (X_i - X_0)/\Delta X \quad (3)$$

Where x_i is the i^{th} coded value of the variable X, X is the variable A, B or C, X_i is the i^{th} natural value of X, X_0 is the centre value of X and ΔX is the step change value of X. The range and levels of the factors varied are given in the Table 1.

Six star points and six centre point replicates were employed in the design for the fitting. The star points and the factorial points were done in triplicate, thus requiring a total of 48 experiments. All experiments were carried out in one run using a single microtiter plate with immobilized oxygen sensors, thus reducing the blocking effects. The central points were taken as those in which the cells were known to be viable, and in which the stock cells were prepared, i.e. the concentrations in the chemically defined medium used for the cell growth. These concentrations were designated as 100% and step changes were applied. The tabulation of the experimental variations and the observed response are given in Table 2.

Cell growth and oxygen uptake rate (OUR) determination

The cell inoculum was grown in the chemically defined medium in spinner flasks with CO_2

Table 1. Range and levels of the factors varied in optimization experiments.

Coded values	Inorganic salts (A) (%Conc.)	Glutamine (B) (%Conc.)	Glucose (C) (% Conc.)
− 2	33.33	33.33	33.33
− 1	66.66	66.66	66.66
0	100	100	100
1	133.33	133.33	133.33
2	166.66	166.66	166.66
ΔX	33.33	33.33	33.33

%Concentration refers to the concentration as a percentage used in the original medium.

Table 2. Medium formulations according to the statistical design with observed and predicted response (at 72 h). The predicted response is obtained from the regression model developed.

Run no.	A	B	C	Observed response OUR ($\times 10^{-6}$ M h^{-1})	Predicted response OUR ($\times 10^{-6}$ M h^{-1})
1	−1	−1	−1	0.093	0.11
2	−1	−1	−1	0.119	0.11
3	−1	−1	−1	0.109	0.11
4	1	−1	−1	0.093	0.085
5	1	−1	−1	0.062	0.085
6	1	−1	−1	0.096	0.085
7	−1	1	−1	0.119	0.13
8	−1	1	−1	0.137	0.13
9	−1	1	−1	0.132	0.13
10	1	1	−1	0.149	0.15
11	1	1	−1	0.145	0.15
12	1	1	−1	0.142	0.15
13	−1	−1	1	0.144	0.14
14	−1	−1	1	0.140	0.14
15	−1	−1	1	0.134	0.14
16	1	−1	1	0.138	0.12
17	1	−1	1	0.095	0.12
18	1	−1	1	0.135	0.12
19	−1	1	1	0.143	0.16
20	−1	1	1	0.153	0.16
21	−1	1	1	0.16	0.16
22	1	1	1	0.135	0.12
23	1	1	1	0.125	0.12
24	1	1	1	0.1	0.12
25	−2	0	0	0.023	0.051
26	−2	0	0	0.09	0.051
27	−2	0	0	0.048	0.051
28	2	0	0	0.014	0.021
29	2	0	0	0.005	0.021
30	2	0	0	0.043	0.021
31	0	−2	0	0.16	0.15
32	0	−2	0	0.159	0.15
33	0	−2	0	0.16	0.15
34	0	2	0	0.17	0.16
35	0	2	0	0.166	0.16
36	0	2	0	0.17	0.16
37	0	0	−2	0.15	0.15
38	0	0	−2	0.155	0.15
39	0	0	−2	0.153	0.15
40	0	0	2	0.161	0.16
41	0	0	2	0.158	0.16
42	0	0	2	0.156	0.16
43	0	0	0	0.168	0.16
44	0	0	0	0.160	0.16
45	0	0	0	0.162	0.16
46	0	0	0	0.155	0.16
47	0	0	0	0.160	0.16
48	0	0	0	0.143	0.16

Run 1–24 represent the Factorial Design, Run 25–42 are the Star points and Run 43–48 are the Central points.

incubation and provided with additional buffering by the addition of 35 mM HEPES. All different medium compositions used for the study were prepared with 35 mM HEPES buffering to carry out the cultivation without CO_2 incubation and a lower concentration of sodium bicarbonate. Cells in growth phase were harvested and equal volume (containing equal no. of viable cells) were transferred to 1.5 ml tubes, centrifuged and re-suspended in the various medium compositions to be tested to give a final viable cell concentration of 1×10^5–2×10^5 cells/ml. This ensured that the final viable cell concentration inoculated into each medium formulation was the same. 200 μl of these were transferred to the wells of the microplate, covered with a lid and cultivated in the fluorescence reader at 37 °C and 660 rpm (orbital) with a shaking diameter of 2 mm. The plate was read every 30 min for a cultivation period of 72 h. The outer wells of the plate were not used for cultivation and were filled with water to reduce evaporation. All other wells were used for the experimentation which included the calibration points of the plate. At the end of 72 h, it was seen that the evaporation from the wells varied from 25 μl at the well designated as B11 (outermost used for the growth experiment) to 15 μl at the innermost well (D6). The evaporation was the highest in the outermost wells (A1, A2, A12 etc), which were not used for the study. This uneven evaporation across the plate, which was always below 15%, is one of the limitations of the system leading to variable osmolalities in different wells. However, it has been shown that an increase of up to 20% in osmolality has no effect on growth rate in case of CHO cells (Kimura and Miller 1996).

Oxygen uptake rate was estimated for each the well using a stationary liquid phase O_2 balance (John et al., 2003),

$$\frac{d[O_2]_{aq}}{dt} = k_L a\left([O_2]^*_{aq} - [O_2]_{aq}\right) - \text{OUR} \qquad (4)$$

where $[O_2]$ and $[O_2]_{aq}^*$ are the dissolved oxygen concentrations in the liquid phase and in equilibrium with the gas phase, respectively, and $k_L a$ is the volumetric liquid phase mass transfer coefficient. $[O_2]_{aq}$ was measured using the oxygen sensor coated microplate in conjunction with the fluorescence reader. The saturation oxygen concentration $[O_2]_{aq}^*$ is 0.21 mM, which is the solubility of oxygen in water at 37 °C. The $k_L a$ was determined experimentally as 0.9 h^{-1}, for the cultivation conditions, using sodium dithionite (Fluka Chemie AG, Buchs, Switzerland).

Results and discussion

On-line oxygen profile and oxygen uptake rate

The oxygen uptake rates of the cells, measured using the oxygen sensor coated microplate, are directly related to the culture viability (Deshpande and Heinzle 2004). The online dissolved oxygen profiles obtained from Equation (2) are converted to the oxygen uptake rates by using Equation (4). Figure 1 shows both the dissolved oxygen profile as well as the uptake rate of cells grown in two different medium formulations. Figure 1a shows

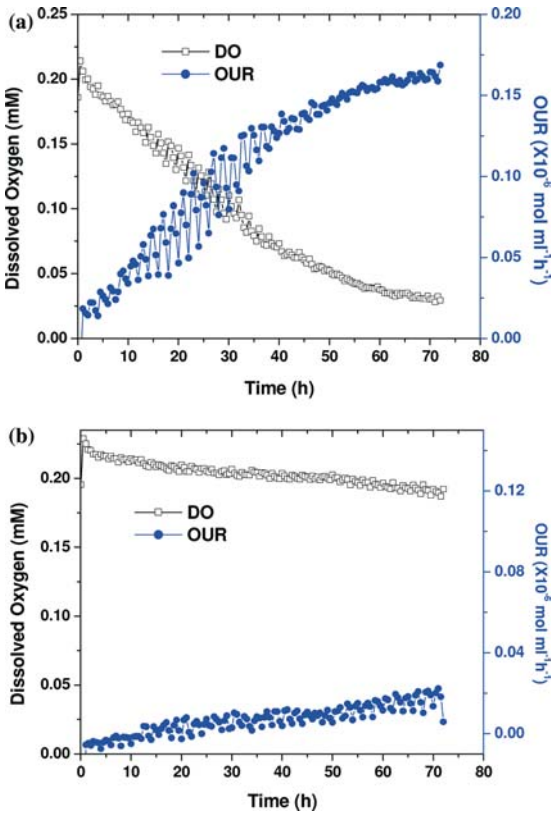

Figure 1. The dissolved oxygen and oxygen uptake profile of two medium formulations. (a) Medium formulation with compositions known to support viability (A = 0, B = 0 and C = 0), (b) Medium formulation with the higher amount of the inorganic salt mixture (A = 2, B = 0 and C = 0). Concentration levels A, B and C are specified in Table 1.

the profile and uptake for the medium formulation with the coded values A = 0, B = 0 and C = 0. Figure 1b shows the same with the medium formulation of coded values A = 2, B = 0 and C = 0. From the figure it can be seen that there is a marked difference in the profiles of both, with the former showing a higher final oxygen uptake than the latter. This effect is most probably due to the presence of one or several components in toxic concentrations. Thus by comparing the two profiles a preliminary idea on the effect of different compositions can be obtained. From the figure it is clear that a high concentration of inorganic salt mixture causes a decrease in cell vitality. Also potential components limitations could be identified with the 72 h oxygen profiles.

The final oxygen uptake obtained for each formulation after 72 h is listed in Table 2.

Development of regression equation

The experimentations were carried out using the 2^3 full factorial central composite design. Design Expert 6.0 (Stat Ease Inc., Minneapolis, USA) was used for the regression analysis. A second order polynomial model (which includes the linear, quadratic and the interaction terms) is generally adequate to describe the system. But this was found to have a lack of fit with an F-value of 3.14 (Massart et al. 1997). So a reduced third order model was applied. The model is described by the equation

$$\begin{aligned} \mathrm{OUR} = {} & b_0 + b_1 * A + b_2 * B + b_3 * C + b_4 \\ & * A^2 + b_5 * AB + b_6 * AC + b_7 * BC \\ & + b_8 * A^2B + b_9 * A^2C + b_{10} * ABC \end{aligned} \quad (5)$$

Where A is the concentration of inorganic salt mixture, B is the concentration of glutamine and C is the concentration of glucose. Also b_0 is the intercept coefficient and $b_{i\ =\ 1\ \ldots 6}$ are the coefficients referring to the measures of effect of the various variables involving A, B, and C. This model was not aliased (confounded) and therefore could be applied. The analysis of variance (ANOVA) of the model demonstrates that the model is significant (Table 3), as is evident from the Fisher F test (F_{model} = 42.20) and a low probability of failure (''Prob > F'' = < 0.0001) (Massart et al. 1997). The ''Lack of Fit F-value'' of 0.52 implied that the Lack of Fit was not significant relative to the pure error. The goodness of fit was checked by the determination coefficient (R^2). For this analysis, the value of the determination coefficient (R^2 = 0.92) showed that there was a good agreement in the model and the responses. The adjusted determination coefficient (adj. R^2 = 0.856) indicated significance of the model too. The application of the methodology yielded the following equation with the estimated coefficients,

Table 3. ANOVA for the response surface reduced cubic model.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob > F	
Model	0.079	10	7.887×10^{-3}	42.20	< 0.001	Significant
A	2.71×10^{-3}	1	2.71×10^{-3}	14.51	0.0005	Significant
B	1.04×10^{-4}	1	1.04×10^{-4}	0.55	0.4611	
C	5.95×10^{-5}	1	5.95×10^{-5}	0.32	0.5759	
A^2	0.067	1	0.067	357.32	< 0.001	Significant
AB	2.47×10^{-4}	1	2.47×10^{-4}	1.32	0.2580	
AC	5.65×10^{-4}	1	5.65×10^{-4}	3.02	0.0905	
BC	1.95×10^{-3}	1	1.95×10^{-3}	10.43	0.0026	Significant
A^2 B	1.18×10^{-3}	1	1.18×10^{-3}	6.33	0.0164	Significant
A^2 C	6.46×10^{-4}	1	6.46×10^{-4}	3.45	0.0710	
ABC	1.09×10^{-3}	1	1.09×10^{-3}	5.81	0.0210	Significant
Residual	6.92×10^{-3}	37	1.87×10^{-4}			
Lack of fit	4.13×10^{-4}	4	1.03×10^{-4}	0.52	0.7189	Not significant
Pure error	6.50×10^{-3}	33	1.97×10^{-4}			
Cor total	0.086	47				

The Model F-Value of 42.20 implies that the model is significant.
Values of ''Prob > F'', probability of failure, less than 0.05 indicate model terms significant.

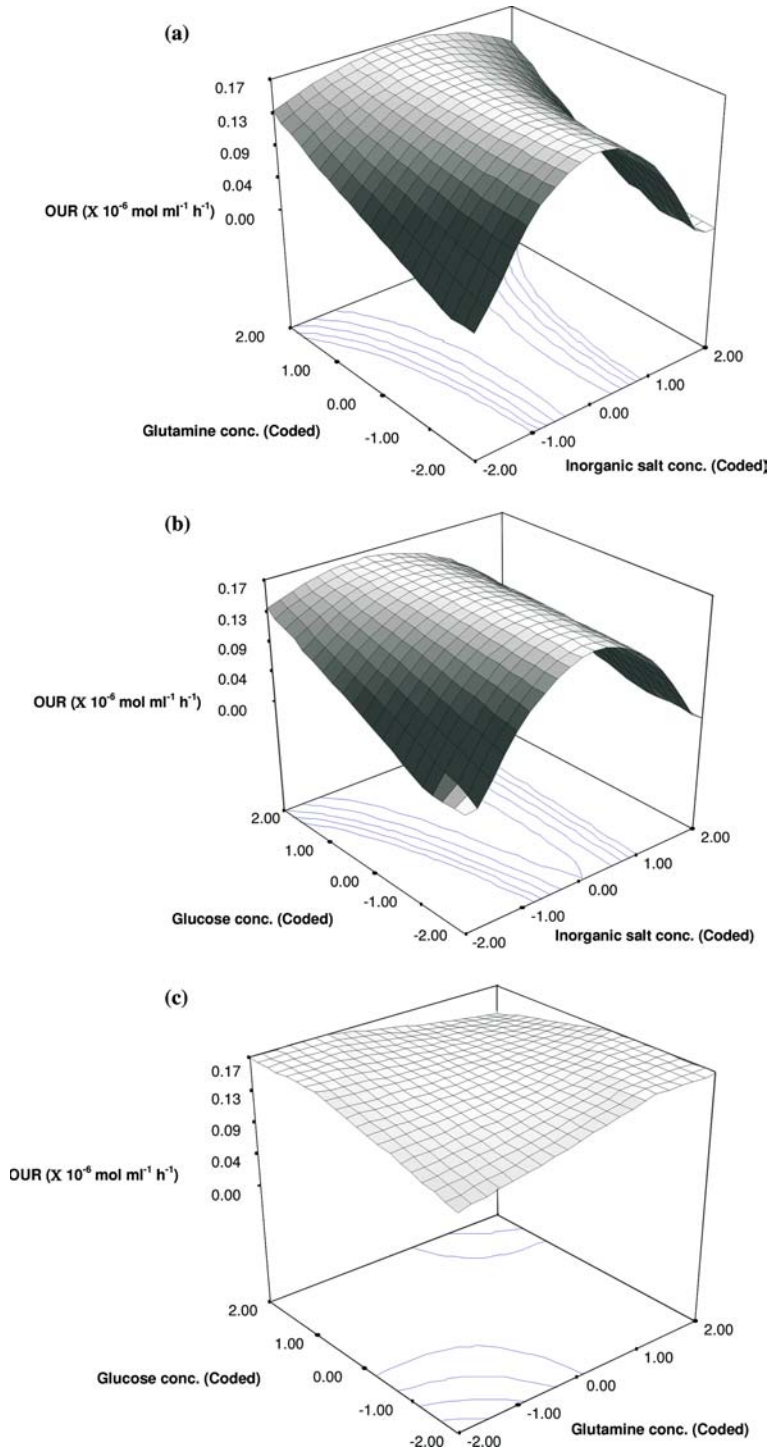

Figure 2. Response surface plot showing effects of medium components on the oxygen uptake and hence the culture viability. All other variables were held constant. Concentration levels are specified in Table 1. (a) Effect of inorganic salt and glutamine concentrations. (b) Effect of glucose and inorganic salt concentrations. (c) Effect of glucose and glutamine concentrations.

$$\begin{aligned}\mathrm{OUR} = {} & 0.16 - 7.157 * 10^{-3}A + 2.079 \\ & * 10^{-3}B + 1.575 * 10^{-3}C - 0.030A^2 \\ & + 3.206 * 10^{-3}AB - 4.851 * 10^{-3}AC \\ & - 9.011 * 10^{-3}BC + 9.926 * 10^{-3}A^2B \\ & + 7.335 * 10^{-3}A^2C - 6.729 \\ & * 10^{-3}ABC \end{aligned} \quad (6)$$

where the terms have the same notation as in Equation (5). The significance of each coefficient was estimated by the F-value. In this case A, A^2, $B*C$, A^2*B and $A*B*C$ are significant model terms. The predicted response of each of the runs from the model is compared with the observed response in Table 2.

Response surface plots of two variables, keeping the others constant, are useful to understand the influence of main and interacting effects of the factors. Figures 2a, b and c shows the response surface plots of the three tested variables. The salt mixture exerts the most important effect on the rates with no dramatic effects seen by the variation in glucose and glutamine concentrations in the concentration range used. The long term effects of these composition ranges are however not taken into account for this particular set of experimentations.

A growth experiment was performed with the optimized medium composition obtained from the regression analysis, and the composition is shown along with its predicted response in Table 4 and is marked selected. A growth experiment was also performed with a second optimum given by the analysis to check the validity of the model. The oxygen uptake profiles of the compositions in comparison with the original composition are given in Figures 3a and b. It is seen that the proliferation rate of the cells with the new medium was significantly higher than with the original medium in the initial culture phase thus showing the applicability of the method used. Using OUR for earlier growth phases, e.g. 48 h and 60 h, similar optimum concentrations could be obtained with a standard deviation in the concentration of up to about 10%.

Table 4. Optimum medium compositions obtained from the model (Equation (6)).

No.	A-Inorganic salt conc. (%)	B-Glutamine conc. (%)	C-Glucose conc. (%)	Predicted OUR.	Solution
Reduced third order model					
1	117.3	165.9	41.26	0.20	Selected
2	105.7	142.3	52	0.18	

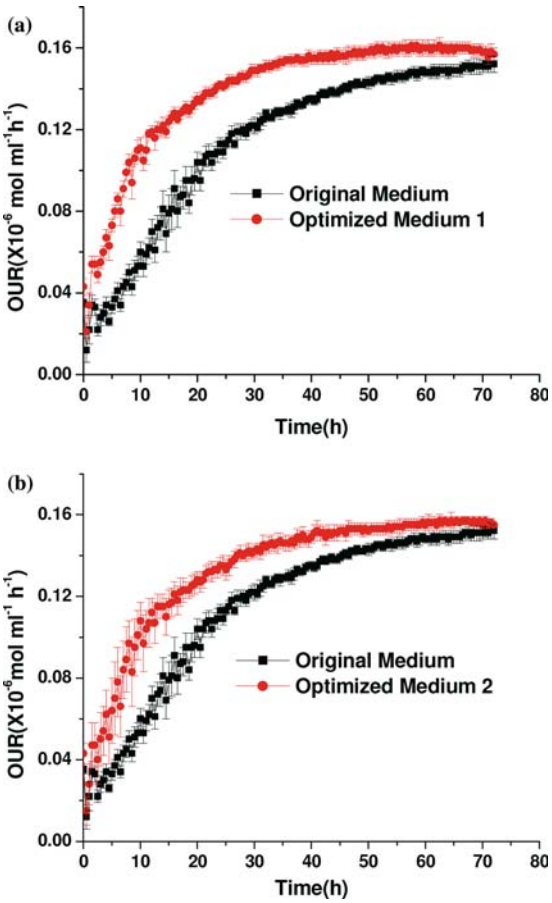

Figure 3. The oxygen uptake profiles of the medium composition obtained from optimization compared with the original composition. (a) Medium formulation with the optimized composition 1 (A = 117.3%, B = 166%, C = 41.26%) compared with the original composition (A = 100%, B = 100%, C = 100%). (b) Medium formulation with the optimized composition 1 (A = 105.7%, B = 142.3%, C = 52%) compared with the original composition (A = 100%, B = 100%, C = 100%).

Conclusions

The above experiments show that microplates coated with oxygen sensors could be used in the primary step of medium optimization. The system is fast, allows high-throughput experiments and

measures the metabolic activity of cells. The two major limitations of the method as presented here are: (i) the limited time for growth experiments (< 100 h) due to the evaporation from the wells, (ii) the fact that the experiments in the present experimental set-up have to be done in externally buffered media with HEPES. The uneven evaporation of medium from the wells of the plates and presence of HEPES could confound the analysis in case of sensitive cell lines. However, both could be overcome by putting the microplate reader into an appropriately humidified chamber with controlled addition of carbon dioxide. Loss of water could also be compensated for by the addition of water after, e.g. 80 h. The method may also be extended by the measurement of the product formed as a secondary output in the statistical analysis. The method is highly cost effective since the amount of medium required is very small for each run, a lot of samples can be analyzed at a single go and the oxygen uptake rate measurements are entirely automatic minimizing labor cost. This method seems directly applicable for almost any culture of suspended and probably also attached animal cells which can be grown in microplates and have comparable oxygen uptake rates as the cells used here. This method seems also useful for other purposes as e.g. test of raw materials and conditions of cell stocks which are considered important in mammalian cell production (Hesse and Wagner 2000).

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