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OPTIMIZATION TECHNIQUES: STUDIES IN CELL CULTURE

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ABSTRACT

With the present large worldwide demand for vaccines and cellular byproducts, a number of anchorage-dependent cell mass culture systems have been developed. To obtain the maximum yield of product per run, the number of cells per system volume must be maximized and yet maintain efficiency. There is a large number of independent variables that could affect the cell yield. During the course of an experimental program to increase the cell yield, the usefulness of an existing computer optimization program was evaluated.

A series of nine statistically designed experiments was done using multi-disk propagators for the mass culture of primary chick embryo cells. Cell input and time between medium refeeds were chosen as the independent variables from the many variables that

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could affect cell yield. The primary dependent variable was cell yield. The results indicated a substantial increase in cell yield as cell input was increased (57 to 85 percent increase by doubling the cell input) but a lesser increase in cell yield with an increasing number of refeeds.

The data were subjected to regression analysis by the existing computer program. The second order polynomial equation (cell output as a function of cell input and time between refeeds) aptly described the experimental data. Three additional experiments were done with 50 percent more cell input than the maximum cell input in the experimental design. The predicted cell yields from the polynomial equation were approximately 10% greater than the cell yields obtained from the three experiments.

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INTRODUCTION

In the field of vaccine production, numerous different types of anchorage-dependent cell propagators have been developed in order to scale up the traditional glass bottle cell culture techniques.[1] They all have in common a large surface to volume ratio and a need to maximize the number of cells per propagator volume in order to produce the highest possible concentration of virus or viral antigen. The maximization of the cell yield is dependent on a large number of propagator operational modes and variables. Any one of the engineering optimization techniques for multivariate systems should be applicable to solving this maximization problem.

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An experimental program was initiated to increase the yield of chick cells in multi-disk propagators[2]. These propagators were developed[3,4,5] for vaccine production[6].

There are many modes of operation and variables that could be considered to have an effect on the propagator cell yield. Among the former are one or two-sided cell planting (initial cell attachment), disks rotated or stationary during the vertical (disks horizontal) plant, sparging or no sparging during the vertical plant, and a horizontal (disks vertical, one-half full of media) or a vertical (disks horizontal) growth cycle. The operation modes that were used in this study are underlined.

The independent variables that were considered for study are cell input (number of chick embryos), percent fetal bovine serum in the medium, temperature, time of plant cycle and growth cycle, disk rotation rate, sparging (bubble size, rate, gas composition), initial medium pH, medium pH during the plant and growth cycles, that is dependent on the sparging, and time between refeeds.

One method of organizing the experimental work to maximize the cell yield is by the use of statistically designed optimization techniques. Based on the results of a statistically designed set of experiments, one can generate relationships between the variables within a system. The independent variables (those under the experimenter's control) and the dependent variables (the experimental results or product properties) are

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mathematically related, and the relationships can be used to select the optimum experimental or manufacturing conditions to achieve the desired results. This may be done by mathematical methods, search methods or graphical analysis.

The purpose of this investigation is to demonstrate the applicability of these statistical techniques to a cell culture growth problem and to illustrate the predictive capability of the equations generated. A previously published technique[7] and an available set of computer programs in these laboratories constituted the basis of this test.

According to the statistical design upon which the technique is based[8], the number of experiments required for these studies is dependent on the number of independent variables studied. For simplicity, it was decided to perform a two variable study which requires a predetermined set of nine experiments. The two independent variables selected from the preceding list were the time between refeeds (X_1) and the number of embryos (X_2). These were chosen because prior experimentation had indicated that they would produce a significant effect on the yield; i.e., more cells should be obtained when more primary cells are seeded, if sufficient medium is provided. In addition, they are both amenable to control and change in a production situation. It is also important to note that, except for the sparged gas composition which was changed to decrease the amount of change in the medium pH during growth, the other variables listed were held

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constant throughout the study. The dependent variables selected were the harvested cell yield (Y_1) and glucose utilization (Y_2). The number of dependent variables is unlimited since each is treated separately, but no others were selected for this preliminary study.

This is the first application of the pharmaceutical formulation computer optimization program of Schwartz et al.[7,9] to a mass cell culture experimental problem. This program was designed to handle multivariate systems with two to five independent variables and any number of dependent variables. The experimental design is a modified full or half-factorial set of experiments (depending on the number of independent variables). The relationships between the dependent and independent variables are generated by regression techniques and expressed as second order polynomial equations of the following form:

$$Y_i = A_o + A_1 X_1 \ldots + A_n X_n + A_{11} X_1^2 \ldots + A_{nn} X_n^2 + A_{12} X_1 X_2 \ldots + A_{(n-1)n} X_{n-1} X_n \quad \text{(Eq. 1)}$$

where: Y_i = the level of the dependent variable

X_i = the level of the independent variable

A_i = the regression coefficients

For the two variable study in this case, the polynomials would have six terms and would take the form:

$$Yi = A_o + A_1X_1 + A_2X_2 + A_{11}X_1^2 + A_{22}X_2 + A_{12}X_1X_2 \quad \text{(Eq. 2)}$$

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If necessary, the dependent variables can be optimized by feasibility and grid search routines. Also, plots can be generated by the computer based on the polynomial equations.

EXPERIMENTAL

Propagators

Four multi-titanium disk propagators as shown in Figure 1 were used. The area of one side of the disk stack is 9,000 cm^2. The volume of medium used in the vertical planting mode (disks horizontal) was 4.2L and the volume in the horizontal (disks vertical) was 2.5L.

Media

A 199 medium[10] was used with 2% fetal bovine serum. The same lot of medium which was kept at 5°C, was used for all experiments and the fetal bovine serum was thawed and added before use.

Cell Suspension

The primary chick cell suspensions were made each week by the same conventional trypsinization procedure which included the use of 11-day chick embryos, trimming off the heads, weighing the trimmed embryos, mincing, trypsinization, centrifugation and resuspension in a 199 medium with 10% fetal bovine serum. The cells per embryo are determined by a Coulter counter[11].

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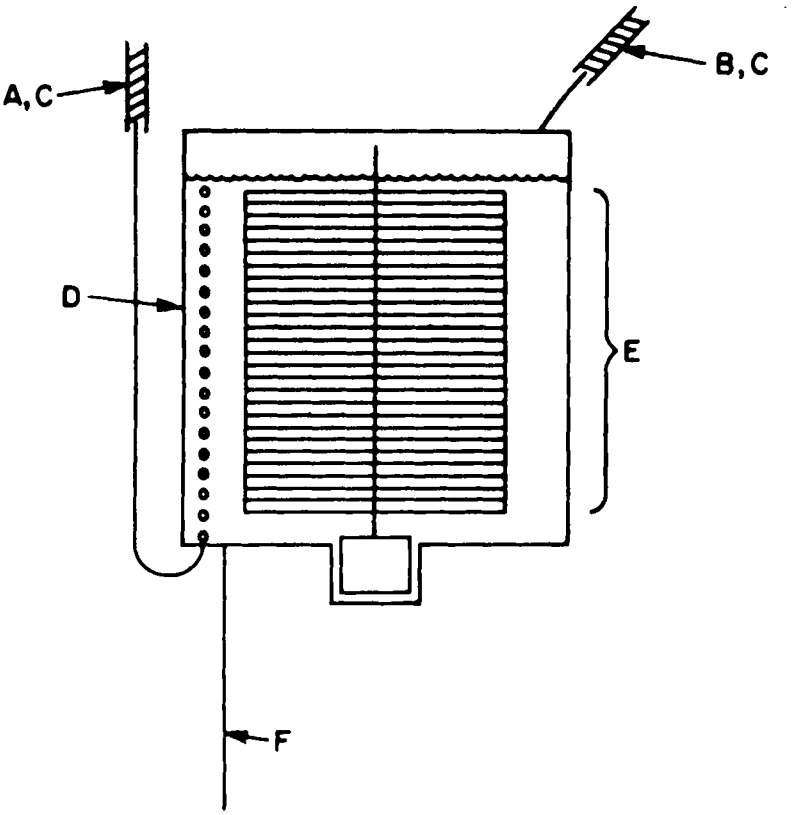

FIGURE 1
Schematic Diagram of Multi-Titanium Disk
Propagator in Vertical Planting Mode
(A) Gas inlet (B) Gas outlet (C) Filters (D) Glass sleeve
(E) 50 disks (F) Inlet/Outlet/Sampling Line

Experimental Procedures

As discussed in the Introduction, a two-independent variable study with two dependent variables was chosen. The independent variable experimental ranges must also be chosen. The independent variables are embryo input (18 to 36 embryo range) and refeed time (12 to 24 hour range). The first refeed was always effected at 43 hours after time of planting for all the experiments and the experiments were all 91 hours in duration, so a range of refeed

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times of 12 to 24 hours would require a maximum of 4 refeeds and a minimum of 2 refeeds.

For this situation, the experimental program which uses the statistical design of Box and Wilson[7,8] specifies an expanded full factorial experimental design (nine experiments) as shown in Table I.

The X_1 and X_2 in this table are shown in both experimental and in physical units. The +1.0 and −1.0 values represent the high and low levels as in any factorial design and the 0.0 experimental level represents an intermediate value. The experiments were done in a random fashion: Week 1 - #2, Week 2 - #5 and #9, Week 3 - #7 and #8, Week 4 - #1 and #3, Week 5 - #4 and #6. Medium is put into the propagator three days before the planting day to preheat at 37°C in an incubator and for a sterility check. The prewarmed medium is removed from the propagator on the day of planting, a sample is taken for pH and glucose concentration (Beckman Glucose Analyzer), the cell suspension is mixed with the medium, and the mixture is transferred back into the vertical propagator. A 5 percent mixture of CO_2 in air sparge is initiated at 100 ml/min. At 19 hours from the initiation of planting, the propagator is turned horizontal, medium is drained so that 2.5L are left, and rotation of the disks is commenced at 1/4 revolution per minute. Within this 19 hour planting phase the cells settle out and attach to the disk surfaces. It has been observed that the complete top surface of the disks is covered with cells. At 26 hours, the CO_2 in air is adjusted to 1 percent to promote

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TABLE I

Experimental Design

	X_1 = Refeed Time		X_2 = Embryo Input	
Run #	Experimental Units[a]	Physical Units, Hours	Experimental Units[b]	Physical Units, Embryos
1	+1.0	24	+1.0	36
2	+1.0	24	-1.0	18
3	-1.0	12	+1.0	36
4	-1.0	12	-1.0	18
5	+1.0	24	0.0	27
6	-1.0	12	0.0	27
7	0.0	18	+1.0	36
8	0.0	18	-1.0	18
9	0.0	18	0.0	27

[a] 1 experimental unit = 6 hours

[b] 1 experimental unit = 9 embryos

control of medium pH. The refeeds were always initiated at 43 hours by draining the medium from the propagator completely and adding 2.5L of fresh preincubated medium. The glucose utilization (Y_2) was calculated by multiplying the initial glucose concentration by the volume of medium for each segment of the experiment and summing all the products.

The propagators were harvested at 91 hours using a standard trypsinization procedure and the cell yield (Y_1) was determined by Coulter counting. Bellco 7000 roller bottle controls to check the

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medium and cell suspension quality and reproducibility were planted each week with a volume of cell suspension equal to one-half embryo each and with the same medium lot used in the propagator experiments. As a convenience these were harvested 24 hours earlier.

RESULTS AND DISCUSSION

The experimental results are summarized in Table II. Three additional experiments (Run #10, 11, 12) were done with a greater cell input. It is seen that there was relatively little week-to-week variation in the cell suspension (cells per embryo) or the Bellco cell yield. Only Runs #4 and #6 had a lower number of cells (20 percent) per embryo and Bellco cell yield apparently due to a lighter than average embryo weight. The cell yield for Run #9, 3.1×10^9 cell input (27 embryos) and 3 refeeds, appears to be low and is probably due to neglecting the sparge for the last 12 hours of the experiment.

Figures 2 and 3 are typical plots of glucose concentration versus time from which the glucose utilization can be calculated. The glucose utilization rate is dependent on a number of factors, including the number of actively growing cells.[12] Figures 2 and 3 show that the glucose utilization rate increases with time implying increasing number of cells. Indeed a cell growth curve for the propagators used in this study shows an initial cell loss within 24 hours and then exponential growth with approximately a 24 hour doubling time for the next 48 hours (unpublished data). The value of approximately 0.9 mg glucose/10^6 cells/day has been used to

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TABLE II
Experimental Results

Run #	Cell Suspension Emb. wt. g	Cells/Emb. x 10^{-6}	X_2 # of Embryos	Cells In x 10^{-9}	X_1 Refeed Time,hr.	# of Refeeds	Y_1 Cell Yield x 10^{-9}	Y_2 Glucose Utilized g	Bellco Control Cell Yield x 10^{-6}
1	1.6	116	36	4.2	24	2	6.9	4.1	106
2	1.6	128	18	2.3	24	2	4.4	3.6	96
3	1.6	116	36	4.2	12	4	7.4	4.6	106
4	1.2	96	18	1.7	12	4	4.0	4.4	84
5	1.7	115	27	3.1	24	2	6.1	3.5	96
6	1.2	96	27	2.6	12	4	5.8	4.8	84
7	1.8	116	36	4.2	18	3	7.2	4.9	97
8	1.8	116	18	2.1	18	3	3.9	4.5	97
9(1)	1.7	115	27	3.1	18	3	5.4	3.7	96
10	1.7	114	52	5.9	24	2	7.7	5.3	116
11(2)	1.7	114	52	5.9	12	4	8.8	-	116
12(3)	1.6	116	54	6.3	12	4	8.8	7.0	102

(1) no sparging after last refeed
(2) contaminated after last refeed
(3) 200 ml/min. sparge after plant cycle

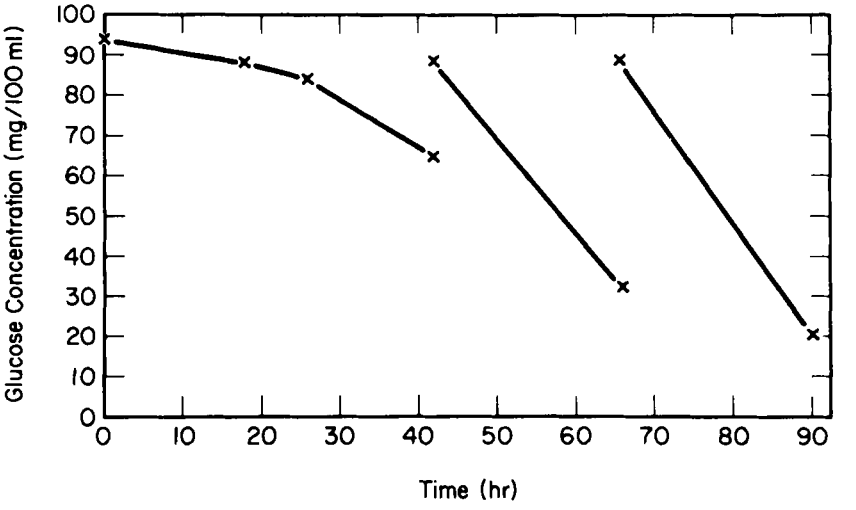

FIGURE 2
Run #2 Glucose Concentration vs. Time plot

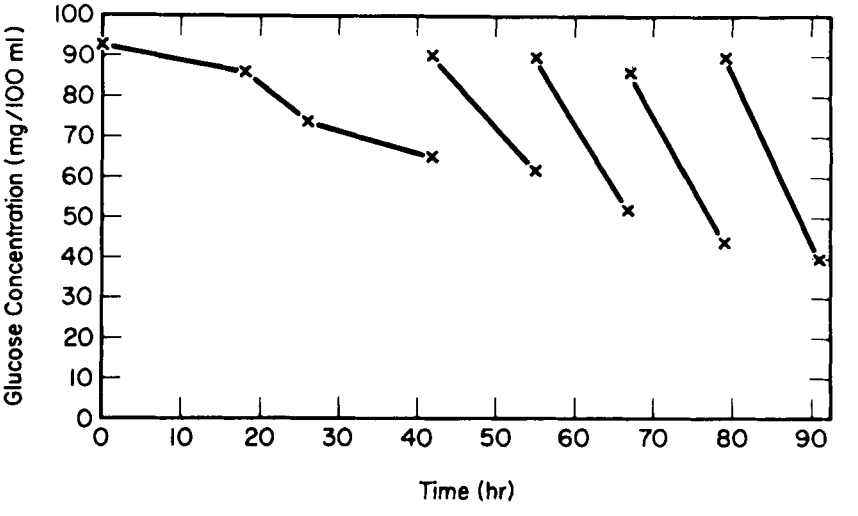

FIGURE 3
Run #3 Glucose Concentration vs. Time plot

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estimate cell number[12]. In Figure 2 for the last 24 hour period a utilization rate per cell of approximately 0.5 mg/10^6 cells/day is estimated based on the average of the final cell yield and one-half that number for the beginning of the 24 hour period. The effect of glucose concentration on growth rate, if any, was not investigated; however, for the conditions used for the nine experiments the glucose was never exhausted.

The cell yields (Y_1) and the glucose utilization data (Y_2) from the nine experiments as shown in Table II were used as input for the computer programs discussed above and the resulting computer generated predictor equations were as follows:

$$Y_1 = 5.589 + 0.033\,X_1 + 1.533\,X_2 + 0.267\,X_1^2 - 0.133\,X_2^2 - 0.225\,X_1X_2 \qquad \text{(Eq. 3)}$$

where: Y_1 = cell output x 10^{-9}

and

$$Y_2 = 4.10 - 0.483\,X_1 + 0.233\,X_2 - 0.150\,X_1^2 + 0.400\,X_2^2 \qquad \text{(Eq. 4)}$$

where: Y_2 = grams of glucose

with R^2 values of 98.9 percent and 82.4 percent, respectively, indicating a good fit. (The R^2 value, or the index of of determination, indicates the fit of the experimental data to the regression equation.) Note that there is no interaction term in Equation 4; i.e., the regression coefficient for the X_1X_2 term was zero. Because of the simplicity of this system, the search programs to maximize cell output were not utilized for this study,

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since those results are apparent from the computer plots and a graphical analysis was sufficient. One can obtain a plot of any dependent variable (Y_i) as a function of any independent variable (X_i) in the study. For example, Figure 4 shows the computer generated plot of cell output as a function of embryo input. Note that the abscissa is presented in experimental units. For this type of plot, the other variable (or variables) must be held constant and in Figure 4 the refeed time is constant at 12 hours (or the -1.0 experimental level). Under these conditions, the cell output (Y_1) increases dramatically with embryo input (X_2), as expected. Note that those experimental points which are applicable have been superimposed on the computer plots.

One can also produce a computer plot of any dependent variable as a function of all independent variables as in Figure 5. For each curve, the other variable (or variables) must remain constant and the appropriate level is indicated on the plot. It can be seen in Figure 5, that when the embryo input is held constant at 27, (or at 0.0 experimental units) varying the refeed time has little effect on the cell output. Increased embryo input, however, does dramatically increase the cell output at a constant refeed time (in this case, 18 hours, or 0.0 experimental units).

A comparison of the embryo input curves in Figures 4 and 5 shows that the slope is slightly increased when the refeed time is more frequent. The effect does not appear to be dramatic. It is obvious that the number of possible plots is unlimited because the

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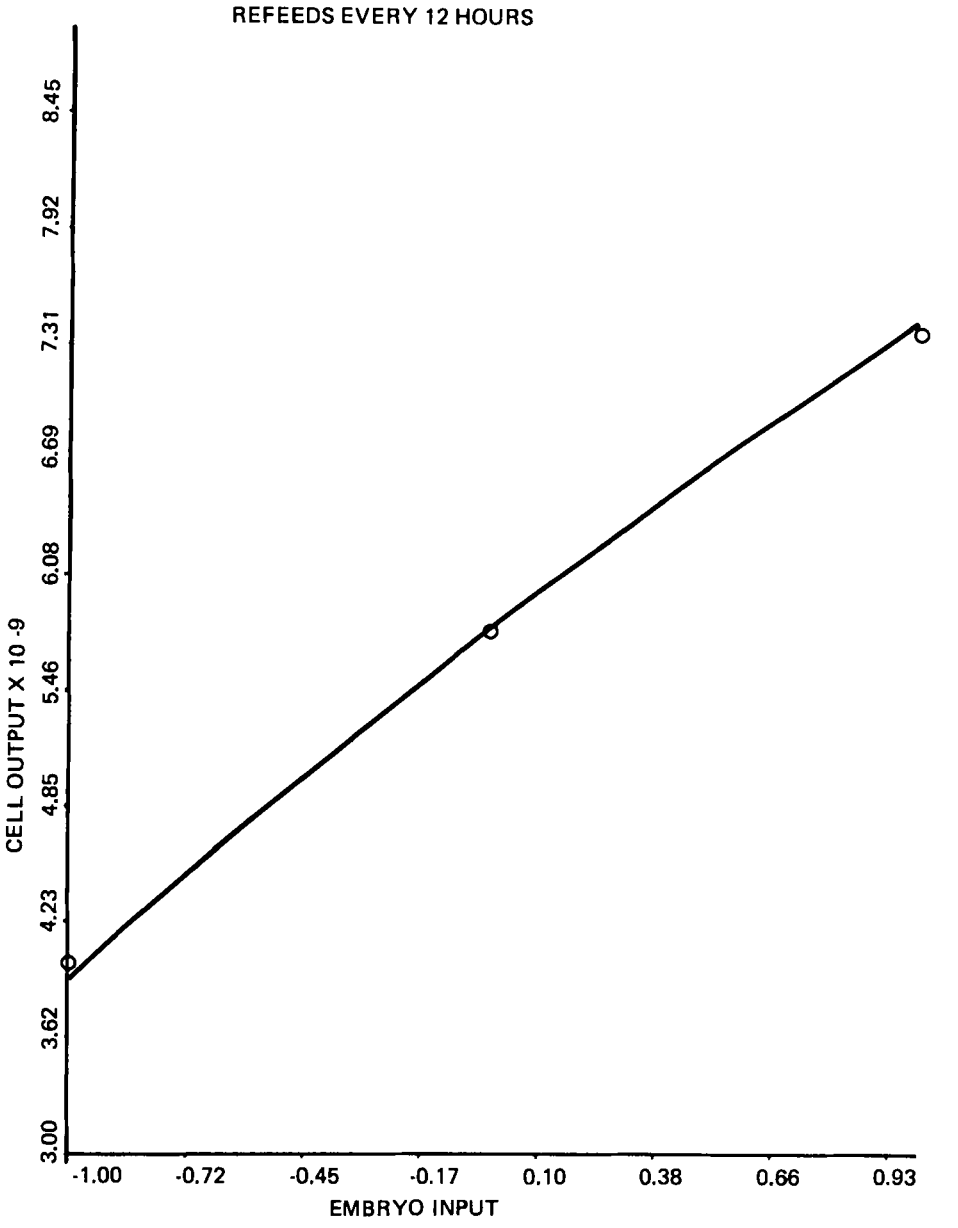

FIGURE 4
Computer plot of Cell Output vs. Embryo Input with Refeeds at 12 hour intervals. 0, experimental points

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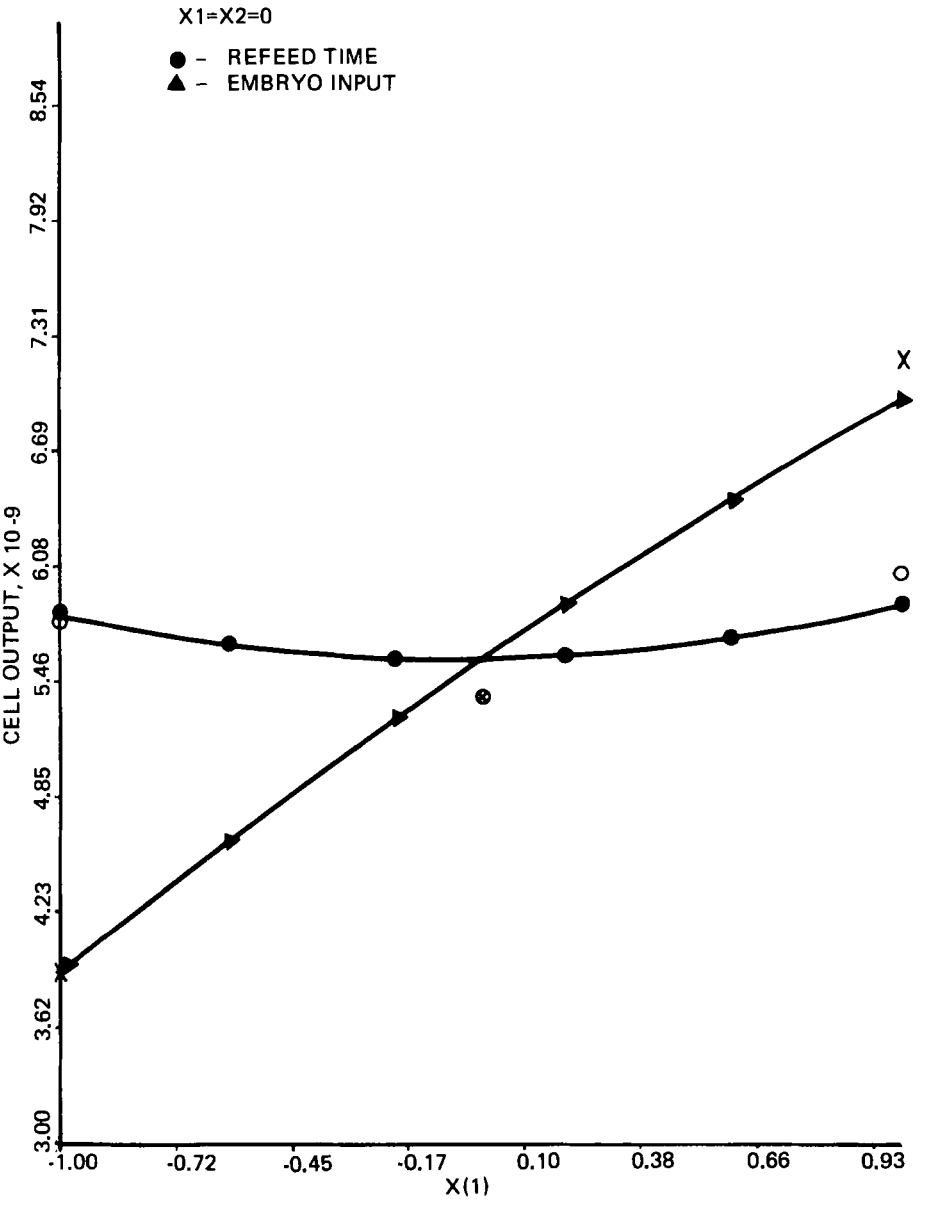

FIGURE 5
Computer plots of Cell Output vs. each Independent Variable. Key O, X experimental points

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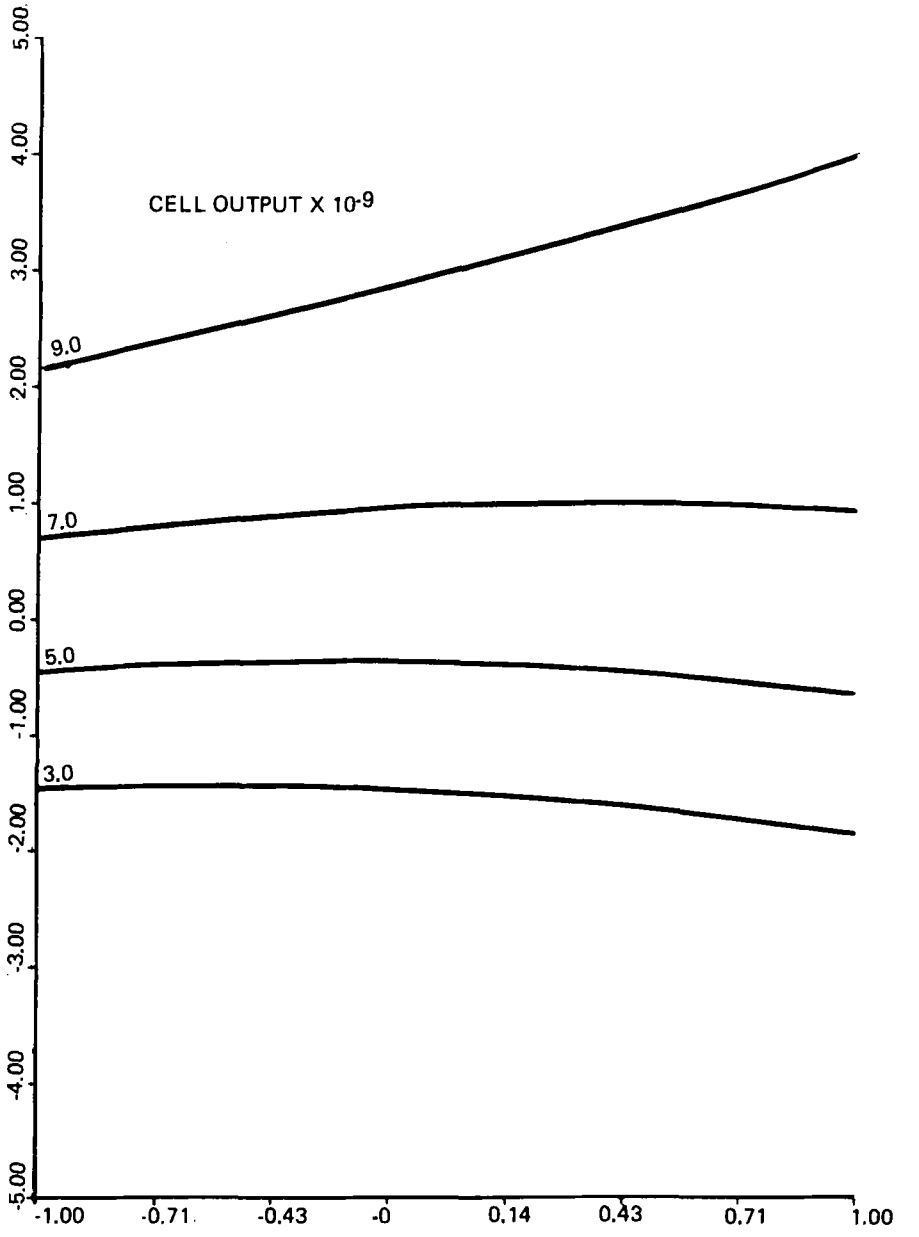

FIGURE 6
Computer contour plots of Cell Output (3.0, 5.0, 7.0 and 9.0 x 10^{-9}) as a function of Embryo Input and Refeed Time

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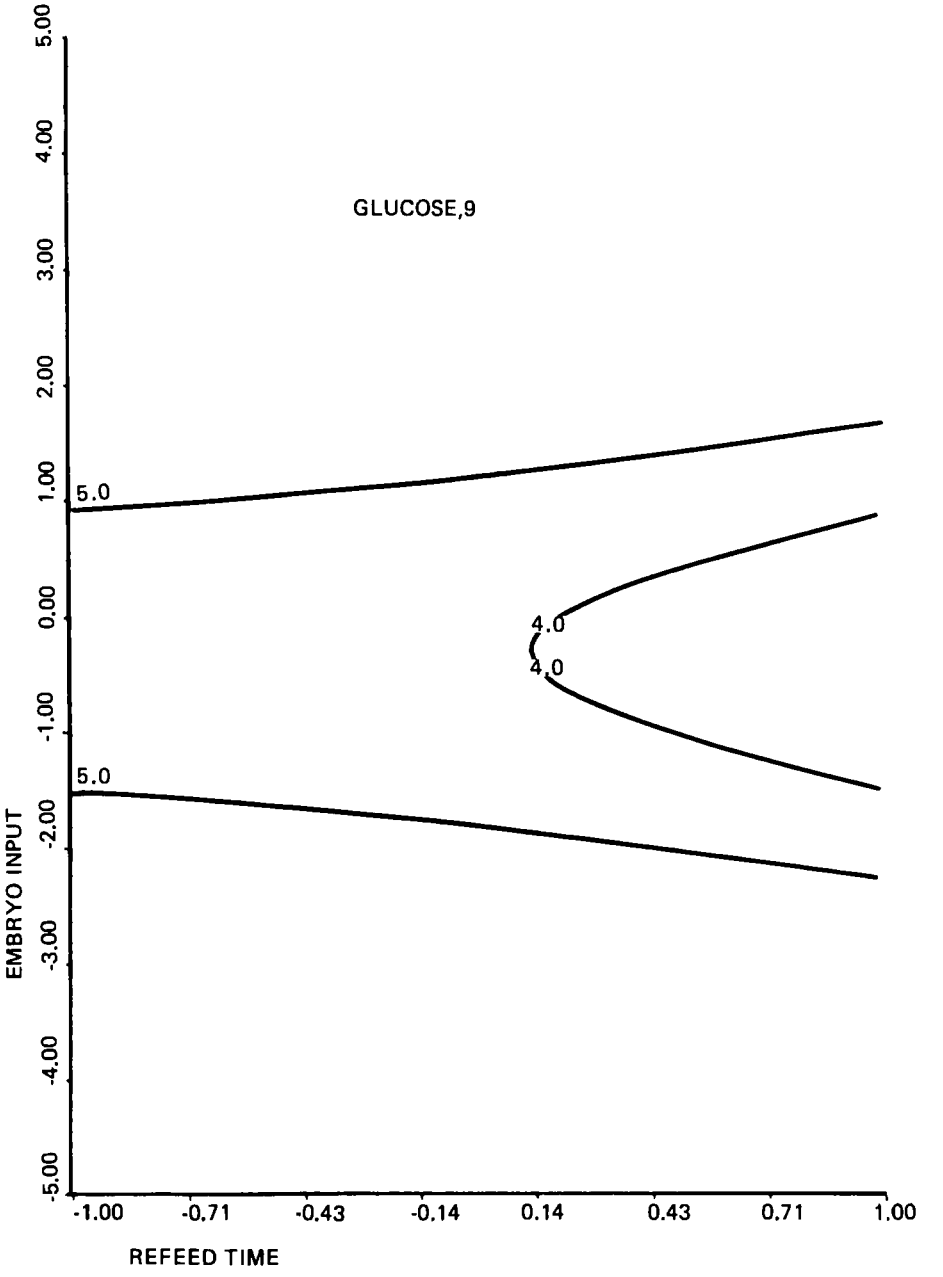

FIGURE 7
Computer contour plots of Glucose Utilization
(4.0 and 5.0 g) as a function of Embryo Input and Refeed Time

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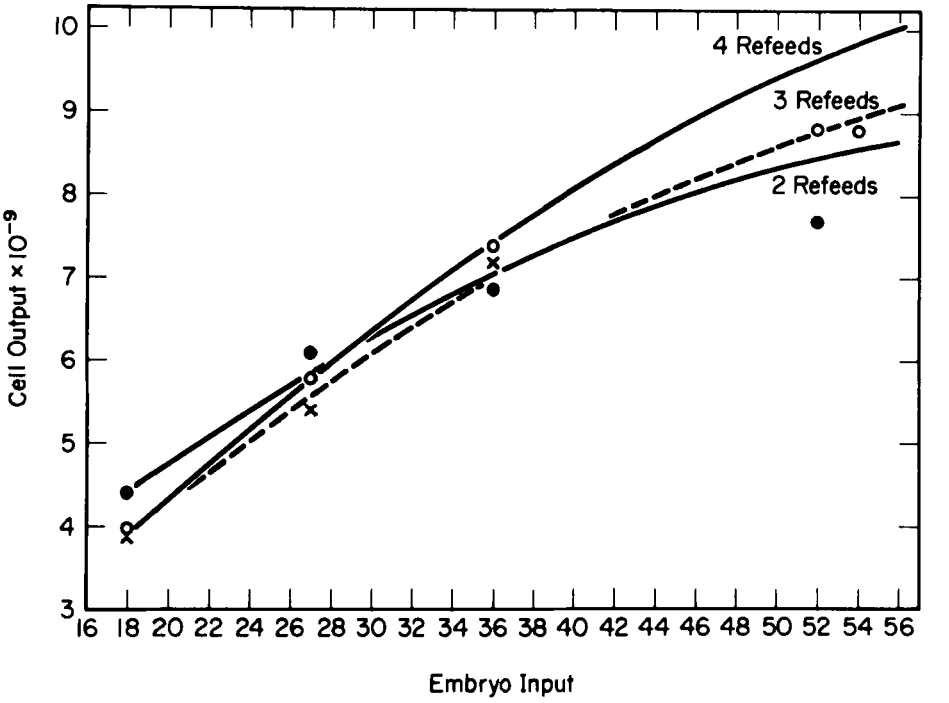

FIGURE 8
Cell Output vs. Embryo Input plot. ●,2 refeeds; x, 3 refeeds; o, 4 refeeds; experimental points. Curves are predicted values from equation 3.

variables may be held constant at any point within the experimental range.

One additional type of plot available is the contour plot (see Figures 6 and 7). In this case, both axes are presented in experimental units for the two independent variables, and the contours represent a given level of the dependent variable represented. Figure 6 represents the cell output and Figure 7 represents the glucose utilization as a function of both X_1 and X_2. With the types of curves presented above, one can obtain a general representation of this system.

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Although predictive capability is not usually expected beyond the experimental range, the computer results have been extrapolated in this case. Figure 8 shows the calculated curves from Equation 3 of cell yield versus embryo input for 2, 3 and 4 refeeds. The calculated curves have been extended from the embryo limit of the nine optimization experiments in Table I to an increased input of 54 embryos. In spite of this 50 percent extrapolation, Equation 3 predicts cell yields only approximately 10 percent higher than the cell yields of experiments #10, 11 and 12, all of which had a high embryo input (see Table II).

CONCLUSIONS

From the above results, it is clear that the two variables that were chosen for study had a significant effect in increasing the cell output, as expected. Cell output was increased from 4.0 x 10^9 to 7.4 x 10^9 cells or 85 percent (18 to 36 embryo input with 4 refeeds) and from 4.4 x 10^9 to 6.9 x 10^9 cells or 57 percent (18 to 36 embryo input with 2 refeeds).

Also it is concluded that the generated second order polynomial equations aptly describe the experimental data and that the statistically based optimization technique is applicable to the mass cell culture problem.

Based on this preliminary study, it is suggested that such techniques can be applied to a more complicated form of this type of problem; i.e., where more than two variables can be studied simultaneously. The results of such studies can be used in cases

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where the effects of changes in the independent variables are not as apparent as they are in this test case, and where interactions between variables are often hidden.

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