

Full Length Research Paper

Optimization and modeling of cellulase protein from *Trichoderma reesei* Rut C30 using mixed substrate

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Bioethanol from cellulosic raw material has proved to be the best alternative renewable energy source. Cellulase is a multienzyme complex catalyses the bioconversion of cellulose to glucose, which can be used for ethanol production. The objective of this research is to reduce the cost of cellulase production by optimization of fermentation conditions and modeling of the fermentation process. Research surface methodology was suggested for optimization of process conditions of cellulase biosynthesis. Logistic kinetic model was the best model for the mixed substrates. A conceptual Artificial Neural Network (ANN) model was well incorporated in the fermentative production of cellulase. By adopting these models high yield of cellulase was obtained.

Keywords: Cellulose, Lactose, Cellulase, Optimization, Modeling, Response surface methodology, Logistic model, Artificial Neural Network

INTRODUCTION

Cellulosic material is the most abundant renewable carbon source in the world. Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for the production of ethanol (Olsson and Hagerdahl, 1996). Cellulase production is the most expensive step during ethanol production from cellulosic biomass, accounting for approximately 40% of the total cost (Muthuvelayudham and Viruthagiri, 2004). Considerable progress has been made in strain development, optimization of culture conditions (Muthuvelayudham et al., 2003), mode of cultivation (Muthuvelayudham et al., 2005) and modeling the fermentation process. The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses (Cochran and Cox, 1957) Combinatorial interactions of medium components especially for mixed substrate namely cellulose with lactose (Muthuvelayudham et al., 2006) with the production of the desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure. Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has

successfully been used in the optimization of bioprocesses (Hao et al., 2006).

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. For some batch processes which have long operating times in each cycle and depend strongly on the operating variables, it was very important to define the optimum conditions in order to achieve sufficient profitability. Kinetic model describing the behavior of microbiological systems can be a highly appreciated tool and can be reduce tests to eliminate extreme possibilities (Lin and Tanaka, 2006). The objective of this work is to apply RSM to evaluate the effects of the medium parameters on cellulase production by the mutant *Trichoderma reesei* Rut C30 and to evaluate the kinetic model for attaining a higher cellulase yield.

EXPERIMENTAL DESIGN AND OPTIMIZATION

Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria. A prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving a more realistic model. A_2^{6-2} fractional factorial designs (FFD) was used to pick factors that influence cellulase production signifi-

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cantly and insignificant ones were eliminated in order to obtain a smaller, more manageable set of factors. In FFD, the range and the levels of the variables investigated in this study are given in the central values (zero level) chosen for experimental design were temperature 28°C (X_1), cellulose 5 g/L(X_2), lactose 5 g/L(X_3), pH 4 (X_4), inoculum's age 5days (X_5) and agitator speed 220 rpm (X_6). In developing the regression equation, the test variables were coded according to the equation:

$$X_j = (Z_j - Z_{0j}) / \Delta_j \quad \text{----- (1)}$$

Where X_j is the coded value of the independent variable, Z_j is the real value of the independent variable, Z_{0j} is the value of the independent variable on the centre point and Δ_j is the step change value. The linear model observed is expressed as follows:

$$Y = \beta_0 + \sum_{j=1}^3 \beta_j X_j \quad \text{----- (2)}$$

Where Y is the predicted response, X_j are input variables which influence the response variable Y ; β_0 is the intercept; β_j is the j th linear coefficient.

If the mean of the center points exceeds the mean of factorial points, the optimum would be near or with the experimental design space. If the mean of the centre points was less than the mean of the factorial points, the optimum would be outside the experimental design space and the method of the steepest ascent should be applied. The direction of the steepest ascent is parallel to the normal contour line of response curve of the model (Equation 1) and passes through the center point of FFD. Increment is direct ratio to regression coefficients β_j . Experiments were performed along the steepest ascent path until the response did not increase any more. This point would be near the optimal point and can be used as center point to optimize the medium parameters.

Once critical factors were identified via screening and significant gross curvature was detected in the design space, the central composite design was proceeded obtain a quadratic model, consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate the pure process variability and reassess gross curvature, with cellulose production as response. For two factors, the model obtained was expressed as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_{12} \quad \text{----- (3)}$$

Where y was the measured response, β_0 is the intercept term, β_1 and β_2 were linear coefficients, β_{12} is the logarithmic coefficient, β_{11} and β_{22} were quadratic coeffi-

cients, and X_1 and X_2 were coded independent variables. Low and high factor settings are coded as -1 and 1, the midpoint coded as 0. The factor settings of trails that ran along axes drawn from the middle of the cube through the centers of each face of the tube are coded as 1.414 or 1.414. The SPSS software, version 10.25 was used for regression and graphical analyses of the data obtained by ridge analysis and analyzing the contour plots. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA).

MODELS FOR GROWTH KINETICS

Monod model

Monod model relates the specific growth rate μ and an essential substrate concentration and was described by equation (Monod, 1949):

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (1)$$

Monod growth kinetics was developed for the carbon substrates, namely lactose and cellulose. The model equation also holds good for non-synthetic substrates namely rice straw and bagasse Monod constant K_s and maximum growth rate μ_{\max} were also calculated for mixed substrates (Muthuvelayudham and Viruthagiri, 2006).

Logistic model

Under optimal growth conditions and when the inhibitory effects of substrates and product play no role, the rate of cell growth kinetics is given by

$$\frac{dX}{dt} = \mu_0 X \quad (2)$$

Where μ_0 is a constant defined as the initial specific growth rate equation implies that X increases with time regardless of substrate availability.

In reality the growth of cell was governed by a hyperbolic relationship and the logistic equation is given by

$$\frac{dX}{dt} = \mu_0 \left[1 - \frac{X}{X_{\max}} \right] X \quad (3)$$

The logistic equation was utilized to describe the kinetics of several polysaccharides fermentation systems (Dhanasekar et al., 2002).

Integrating the equation (3) with the initial condition, $X = X_0$ at $t = 0$ gives a sigmoidal variation of $X(t)$ that may empirically represent both an exponential and a stationary phase

$$X(t) = \frac{X_0 e^{\mu_0 t}}{1 - \left(\frac{X_0}{X_{\max}}\right) (1 - e^{\mu_0 t})} \quad (4)$$

The kinetic parameter, μ_0 was determined by rearranging equation (4) as

$$\ln \left[\frac{X_{\max}}{X_0} \right] = \mu_0 t - \ln \left[\frac{\bar{X}}{1 - \bar{X}} \right] \quad (5)$$

Where $\bar{X} = \frac{X}{X_{\max}}$, if the logistic equation describes the data suitably, then plot of $\ln \left[\frac{\bar{X}}{1 - \bar{X}} \right]$ Vs time should give a straight line of slope ' μ_0 ' and intercept $-\ln \left[\frac{X_{\max}}{X_0} \right]$.

Modified logistic model

As shown is equation (3) the specific growth rate linearly decreases with an increase in the cell mass concentration, when X approaches X_m , the specific growth rate approaches zero. A linear relationship between the specific growth rate and the cell mass concentration could be considered as a specific case and it may not be valid for all strains. A modified form of logistic equation was used to describe the cell growth kinetics by introducing an index of the inhibitory effect 'r' which accounts for the deviation of growth from the exponential relationship (Dhanasekar et al., 2003):

$$\frac{dX}{dt} = \mu_0 \left[1 - \left(\frac{X}{X_m} \right)^r \right] X \quad \text{for } r > 0 \quad (6)$$

When $r = 0$ will be a complete inhibition of cell growth

When $r = 1$ equation (6) reduces to logistic model equation

When r ranges between 0 and 1 equation (6) describes a higher degree of inhibition compared to logistic growth.

When $r > 1$ the growth lies between exponential and logistic patterns.

Equation (6) can be written as:

$$\frac{dX}{X[X_m^r - X^r]} = \frac{\mu_0}{X_m^r} dt \quad (7)$$

Equation (7) was integrated by using partial fraction method with the initial condition, $X = X_0 (t = 0)$ gives

$$X_t = \frac{X_0^r e^{\mu_0 t}}{1 - \frac{X_0^r}{X_m^r} (1 - e^{\mu_0 t})} \quad (8)$$

The cell mass concentration with respect to time depends on the initial and final cell mass concentrations, which varies with microorganisms used and fermentation conditions.

MODELS FOR PRODUCT FORMATION KINETICS

Leudeking-Piret model

The kinetics of cellulase protein production was described by Leudeking-Piret model (Luedeking and Piret, 1959) which states that the product formation rate varies linearly with both the instantaneous cell mass concentration (x) and growth rate $\left(\frac{dX}{dt}\right)$ as:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (9)$$

Where α and β are empirical constants that may vary with fermentation conditions. The parameters α and β were determined and proved to be good for non-synthetic substrates and mixed substrates (Muthvelayudham and Viruthagiri, 2006).

Logistic incorporated Leudeking-Piret model

Logistic incorporated Luedeking - Piret model (Weiss and ollis, 1980) is developed by rearranging equation (9),

using equation (3) for $\frac{dX}{dt}$ and equation (4) for X_t gives

$$\frac{dP}{dx} = \alpha + \frac{\beta}{\mu_0 \left(1 - \frac{X}{X_m} \right)} \quad (10)$$

Integrating equation (10) with two initial conditions $X = X_0 (t=0)$ and $P = P_0 (t=0)$

$$\int_{P_0}^{P_t} dP \int_{X_0}^{X_t} \left[\alpha + \frac{\beta}{\mu_0 \left(1 - \frac{X}{X_m} \right)} \right] dx$$

$$P_t - P_0 = \left[\alpha X - \frac{\beta X_m}{\mu_0} \ln \left(1 - \frac{X}{X_m} \right) \right]_{X_0}^{X_t}$$

$$P_t - P_0 = \alpha [X_t - X_0] - \frac{\beta X_m}{\mu_0} \left[\ln \left(1 - \frac{X_t}{X_m} \right) - \ln \left(1 - \frac{X_0}{X_m} \right) \right]$$

Substitute X_t from equation (4) in the above equation and rearranging gives

$$P_t = P_0 + \alpha X_0 \left[\frac{e^{\mu_0 t}}{1 - \left(\frac{X}{X_m}\right)(1 - e^{\mu_0 t})} \right] + \beta \frac{X_m}{\mu_0} \ln \left[1 - \frac{X_0}{X_m(1 - e^{\mu_0 t})} \right] \quad (11)$$

Modified logistic incorporated Luedeking - Piret model

Rearranging (9) using equation (6) for $\frac{dX}{dt}$ and equation (8) for X_t gives modified logistics incorporated Luedeking - piret equation (Dhanasekar et al., 2002)

$$\frac{dP}{dX} = \alpha + \frac{\beta}{\mu_0 \left[1 - \left(\frac{X}{X_m}\right)^r \right]} \quad (12)$$

Integrating equation (12) with two initial conditions, $X=X_0$ ($t=0$) and $P = P_0$ ($t=0$) gives

$$\int_{P_0}^{P_t} dP = \int_{X_0}^{X_t} \left[\alpha + \frac{\beta}{\mu_0 \left[1 - \left(\frac{X}{X_m}\right)^r \right]} \right] dX$$

$$P_t - P_0 = \left[\alpha X - \frac{\beta X_m^r}{\mu_0} \ln \left[1 - \frac{X^r}{X_m^r} \right] \right]_{X_0}$$

$$P_t - P_0 = \alpha [X_t - X_0] - \frac{\beta X_m^r}{\mu_0} \left\{ \ln \left[1 - \frac{X_t^r}{X_m^r} \right] - \ln \left[1 - \frac{X_0^r}{X_m^r} \right] \right\}$$

Substituting X_t from Equation [8] and rearranging gives

$$P_t = P_0 + \alpha \left\{ \frac{X_0^r e^{\mu_0 r t}}{1 - \frac{X_0^r}{X_m^r(1 - e^{\mu_0 r t})}} \right\}^{1/r} - X_0^r + \frac{\beta X_m^r}{\mu_0} \ln \left[1 - \frac{X_0^r}{X_m^r(1 - e^{\mu_0 r t})} \right] \quad (13)$$

MODELS FOR SUBSTRATE UTILIZATION KINETICS

Modified Leudeking-Piret model

The substrate utilization kinetics was given below was a modified form of the Leudeking-Piret model. Substrate consumption depends on the magnitude of three sink

terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The assumed kinetic form was a linear combination of these terms (Weiss and Ollis, 1980).

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} - \frac{1}{Y_{P/S}} \frac{dP}{dt} - K_e X \quad (14)$$

Substituting Equation [9] is Equation [14], the substrate material balance can be rewritten as:

$$\frac{-ds}{dt} = \left[\frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} \right] \frac{dX}{dt} + \left[\frac{\beta}{Y_{P/S}} + K_e \right] X$$

$$\frac{ds}{dt} = -\gamma \frac{dX}{dt} - \eta X \quad (15)$$

Where

$$\gamma = \frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} (gS^{-1} gX^{-1})$$

$$\eta = \frac{\beta}{Y_{P/S}} + K_e (gS^{-1} gX^{-1} h^{-1})$$

Logistic incorporated modified Leudeking-Piret model

Rearranging Equation [15], using Equation [3] for $\frac{dX}{dt}$ and

Equation [4] for X_t gives

$$-\frac{ds}{dX} = \gamma + \frac{\eta}{\mu_0 \left(1 - \frac{X}{X_m} \right)} \quad (16)$$

Integrating Equation [16] with set of initial conditions $X=X_0$ ($t=0$) and $S=S_0$ ($t=0$) gives

$$\int_{S_0}^{S_t} dS = - \int_{X_0}^{X_t} \left[\gamma + \frac{\eta}{\mu_0 \left(1 - \frac{X}{X_m} \right)} \right] dX$$

$$S_t - S_0 = - \left[\gamma X - \frac{\eta X_m}{\mu_0} \ln \left(1 - \frac{X}{X_m} \right) \right]_{X_0}^{X_t}$$

$$S_t - S_o = -\gamma[X_t - X_o] + \frac{\eta X_m}{\mu_o} \left[\ln \left[1 - \frac{X_t}{X_m} \right] - \ln \left[1 - \frac{X_o}{X_m} \right] \right]$$

Substitute X_t from Equation [4] the about equation and rearranging gives

$$S_t = S_o - \gamma X_o \left[\frac{e^{\mu_o t}}{1 - \frac{X_o}{X_m} (1 - e^{\mu_o t})} - 1 \right] - \frac{\eta X_m}{\mu_o} \left[1 - \frac{X_o}{X_m} (1 - e^{\mu_o t}) \right] \quad (17)$$

Modified logistic incorporated modified Leudeking-Piret kinetic model

Rearranging Equation [15] using Equation [3] for $\frac{dX}{dt}$ and Equation [4] X_t gives

$$\frac{dS}{dX} = -\gamma - \frac{\eta}{\mu_o \left[1 - \left(\frac{X}{X_m} \right)^r \right]} \quad (18)$$

Integrating Equation [18] with set of initial conditions $X=X_o$ ($t=0$) and $S=S_o$ ($t=0$) gives

$$\int_{S_o}^{S_t} ds = - \int_{X_o}^{X_t} \left[\gamma + \frac{\eta}{\mu_o \left[1 - \left(\frac{X}{X_m} \right)^r \right]} \right] dX$$

$$S_t - S_o = - \left[\gamma X - \frac{\eta X_m^r}{\mu_o} \ln \left(1 - \frac{X^r}{X_m^r} \right) \right]_{X_o}$$

$$S_t - S_o = -\gamma[X_t - X_o] + \frac{\eta X_m^r}{\mu_o} \left\{ \ln \left[1 - \frac{X_t^r}{X_m^r} \right] - \ln \left[1 - \frac{X_o^r}{X_m^r} \right] \right\}$$

Substituting X_t from Equation [4] and rearranging gives

$$S_t = S_o - \gamma \left\{ \left(\frac{X_o^r e^{\mu_o t}}{1 - \frac{X_o^r}{X_m^r} (1 - e^{\mu_o t})} \right)^{1/r} \right\} - X_o^r \left\{ \frac{\eta X_m^r}{\mu_o} \ln \left[1 - \frac{X_o^r}{X_m^r} (1 - e^{\mu_o t}) \right] \right\} \quad [19]$$

MODELING BY ARTIFICIAL NEURAL NETWORK

The method of artificial neural network (ANN) for modeling combines the approximation capabilities of neural networks with fundamental bioprocess knowledge, is used to develop a mathematical model of this dynamic system.

The feed forward back propagation algorithm with one hidden layer was used in the training of the neural network, based on varying input/ output pair data sets. A well trained neural network can be employed to predict the bioprocess without prior knowledge of the variables interactions (Linko et al., 1997). Experimental data were used to estimate the parameters of the model. The motivation for using ANN is to improve the accuracy and predictive capabilities of kinetic model and this approach was successfully used for cellulase protein production by mutant strains of *T. reesei*. ANN can be successfully used for both cell mass and cellulase protein production.

Research surface methodology was an optimal tool for optimization of medium parameters for cellulase production. Logistic model and Luedeking-Piret model were found to be appropriate model for obtaining kinetic parameters for best evaluation of fermentation process of converting cellulose to cellulase. A conceptual Artificial Neural Network is used to model the production of cellulase.

CONCLUSION

Research surface methodology was proved to be an optimal tool for optimization of medium parameters for cellulase production. Logistic model and Luedeking Piret model were found to be appropriate model for obtaining kinetic parameters for best evaluation of fermentation process of converting cellulose to cellulase. The use of Artificial Neural Network (ANN) model for the prediction of the parameters of cellulase production was found to be valid.

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