Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2012 Vol. 2 (1) January- March, pp. 170-176 /Ionkov et al.
Research Article

STRATEGY TO CONTROL THE PRODUCTION OF RARE ANTICANCER SUBSTANCES IN THE ENDANGERED PLANT *LINUM LINEARIFOLIUM* IN BIOREACTOR

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ABSTRACT
Podophyllotoxin is a plant-derived compound which is used as educt for the semisynthesis of etoposide and tenoposide. But while the demand for these beneficial anticancer compounds is increasing, the supply of natural plant sources is dwindling. The objective of this study is to improve the biomass growth and podphyllotoxin production in *Linum linearifolium* cells for the first time by 2-L stirred tank bioreactor, using automatic control of the specific growth rate in cell cultivation processes. The present work compared three different control methods. The best one for the production of podophyllotoxin in *L. linearifolium* was found to be Model Control Method. The cultivation of *L. linearifolium* in bioreactor, using this control method resulted in biomass accumulation level of 25.7±0.08 g/L (DCW basis) and podophyllotoxin production of 8.64±0.12 mg/L, which represented a 60% increase compared to that of shake flask cultivation.

Key Words: *Plant cell culture, podophyllotoxin, anticancer lignans, Linum lineraifolium, automatic control methods, bioreactor*

INTRODUCTION

The plant-specific secondary products were long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy. Antitumor activity will undoubtedly continue to be the most clinically relevant property of lignans. Podophyllotoxin (PTOX) is a plant-derived compound which is used as educt for the semisynthesis of etoposide and tenoposide. Both antineoplastic drugs are of importance for the therapies and today and in the future will be a high demand for the commercial drug and its precursors for the treatment of leukemia, lung and testicular cancers. But while the demand for these beneficial compounds is increasing, the supply of natural plant sources is dwindling. Many of these plants are difficult to cultivate or are becoming endangered due to over-harvesting (Ionkova, 2010). Lignans occur in many plant species, but only in low concentrations. Some lignans can only be isolated from extremely rare plants. Our recent studies on the diversity of lignans in the genus Linum have recently presents the chemical data obtained for 54 accessions representing 41 different species. Sixty-four different lignans were detected. Their HPLC-ESI/MSMS, complemented by HPLC- UV/DAD data are reported (Schmidt et al. 2010). Since the natural supply is limited, several research groups have explored the possibility of employing plant cell or organ in vitro cultures for the biotechnological production of these compounds as alternative (Ionkova et al., 2007). The production of anticancer compounds, such as lignan podophyllotoxin, by plant in vitro cultures from plant species is reviewed (Ionkova 2007). Cell cultures of different Linum species of section Syllinum are shown to produce considerable amounts of lignans, mainly 6-methoxypodophyllotoxin (MPTOX). Although the both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs.

Recently, we investigated extracts from *Linum lineraifolium* (Linaceae), which accumulate PTOX besides small amounts of MPTOX as well as traces of some other lignans (Vasilev et al. 2008). *L. linearifolium* is now beside *L. album and L. persicum* the third *Linum* species of section Syllinum with PTOX (ca 0.8% DW) as the main lignan. Since PTOX is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos, the accumulation of predominantly

PTOX in this species is especially interesting. Suspension cultures of *L. linearifolium* (Linaceae) an endemic rare plant species, accumulate podophyllotoxin and could therefore serve as an alternative source of this important aryltetralin lactone lignan (Ionkova et al. 2010).

The objective of this study is to investigate and improve the biomass growth and lignan production in *L. linearifolium* cells by stirred tank bioreactor. Using the optimum conditions of the shake flask system, the growth and product formation kinetics was investigated in a 2 L bioreactor. The success of this technology is dependent not only on the discovery of appropriate production plant system, but also from optimization of cultivation with control of various culture parameters. The control of bioprocess is a difficult task due to the challenges associated with bioprocess modeling and lack of on-line measurements. The present work compared three different control methods of 2-L bioreactor to improve the cell growth and podophyllotoxin production by *L. linearifolium* cells.

MATERIALS AND METHODS

Establishment of in vitro cultures

Seeds of *L. linearifolium* (Lindem.) were collected in Bulgaria near the town Pleven in July 2010. Voucher specimens (FAF 0563) are deposited in the herbarium of the Faculty of Pharmacy, Medical University of Sofia. Callus and suspension cultures were established using standard methods (Ionkova et al. 2010). After 3 to 4 weeks, developed callus cells were subcultivated weekly by transferring 3 g cells to 50 ml fresh MS medium with 0.4 mg l-1 naphtylacetic acid (NAA) and 0.1 mg l-1 kinetin (medium Li-MOD) solidified with 1% agar-agar in 300 ml Erlenmeyer flasks. The suspension cultures were placed on a gyratory shaker (100 rpm) in the dark at 25 ^{0}C. Suspensions (3 g fresh wt) were transferred every 30 days into 50 ml fresh medium.

Cultivation of L. linearifolium in 2 L bioreactor

The cells of *L. linearifolium* were cultivated in a 2L laboratory-scale bioreactor using a two-week dark-grown culture as inoculum (5 g/l, DCW basis). The medium used was "Li-MOD" and the bioreactor was operated at 140 rpm at 26°C in the dark for 21 days. The pH of the culture medium was adjusted to 5.6 before autoclaving and controlled at 5.6 by the automatic addition of aq. NaOH (0.5 mol/l) and aq. HCl (0.5 mol/l) during cultivation. A specially designed low-shear impeller (marine-type) was used for gentle agitation to avoid damage to the cells caused by impeller rotation. An air flow rate of 0.5 vvm was maintained to avoid extensive foaming of the culture broth and to prevent cell damage. Samples (10 ml) were collected in duplicate every 3 days to determine DCW of the biomass as well as residual sucrose in the medium and podophyllotoxin concentrations.

Extraction and Quantitative analysis of lignans

Lignans were extracted from powdered plant cell material (200 mg) with MeOH (2 ml) for HPLC analysis as described in (Ionkova et al. 2010). The HPLC determination was performed on a Thermo Quest (Egelsbach, Germany) equipped with a Spectra SYSTEM UV6000LP detector. The ariltetraline lignans were identified by comparison of the retention time and spectra with authentic standards.

Bioreactor equipment

Fermentations were carried out in 2 l jacketed glass vessel applying cultivation for 21 days. Equipment of the vessel includes: sensors for temperature, pH, dissolved oxygen concentration, foam, and speed of stirrer drive system; four integrated peristaltic pumps and one external pump for flow control and feeding. Bioreactor conditions: temperature - 26 ^{o}C, batch mode of cultivation, dissolved oxygen saturation (DO at 50%), marine-type impeller design with low-shear stress, speed 140 rpm. This impeller provides mixing and creates a higher oxygen mass transfer rate (Kla).

Control system

Control system has the following functions: display of all process values via schematic P&ID algorithms, digital calibration of sensors and pump dosing counters indication of sensor parameters, recalibration function of pH-probe, control loops for temperature, stirrer speed, pH, foam level, substrate, pO2 with two stage cascade control, set point profile for substrate pumps.

Data acquisition

Data acquisition system includes the following functions: data collection, data base maintenance, visualization of the process variables by several plotting functions. This software allows starting or

finished process batches, exporting database in appropriate data formants and sample data configuration.

RESULTS AND DISCUSSION

Mass cultivation of plant cells in vitro is a viable alternative for production of high value, low volume phytochemicals. The automatic control of the specific growth rate in cell cultivation processes is one method for improving the productivity and, hence, cost of a process. The present work compared 3 different approaches for control of bioreactor production of podophyllotoxin and establishment of the best one, using batch cultivation method of *L. linearifolium* in stirred tank bioreactor, because this reactors have been the most extensively applied in order to achieve the optimum process parameters. In spite of the fact that stirred tank reactors exert more hydrodynamic stress on plant cells, they have great potential when used with low agitation speed and modified impeller. A low-shear marine-type impeller has been found to be effective for batch cultivation of shear-sensitive *L. linearifolium* cells in stirred tank bioreactor for production of podophyllotoxin. Although batch cultivation strategy has been widely adopted for scale-up of plant cell bioprocesses, it has not always been successful in improving the production of desired metabolites.

Study of Control methods of Bioreactor production of anticancer lignans

The automatic control of the specific growth rate in cell cultivation processes of secondary metabolites production has become a major issue in process control in the recent years. Several control schemes for bioprocess have been investigated up to this date, a good compendium of them can be found in (Roubos 2002). Our study focuses on the design of accurate adaptive control methods, with guaranteed exponential convergence and its relation with the calibration of controller parameters. The manipulation of environmental factors such as pH, temperature, shear stress and O2 supply are the keys to production of plant cell culture processes. This research presents the implementation of optimal control strategies to control a reactor in production of pure product podophyllotoxin with a high concentration using a mathematical model. In this paper tree control methods are designed:

- Model Control Method, where the nonlinear model process is directly embedded in the control law
- Integral forwardstepping control - a novel nonlinear controller with integral function (Kanellakopoulos and Krein, 1993), proposed for high performance parametric variable control systems.
- Optimal control algorithm, based on design methods with the state-space formulation which gives controllers with the linear structure - for example pole placement (Aström 2003).

Model Control Method

Suppose a process is described by equations (1) and (2):

(1) $$\frac{dx}{dt} = f(x,u,d,t)$$

(2) $$y = g(x)$$

Where **x** is the state vector with dimension n, **u** is the manipulated variable and has dimension m, **d** is the vector of disturbances with dimension l**, y** is the vector of measurements with dimension p. In the general case, both **f** and **g** are nonlinear functions. The classical approach in feedback control of bioreactor is to compare a set-point desired for the output with its actual value, in order to form an error signal to be given as input to the controller. In Model Control, this error signal is also formed, but the control objective is expressed in terms of the value of the derivative of the output. The control scheme calculates the manipulated variable vector so that the derivative of the output follows an established pattern. To get the expression of this pattern, it must be considered that, when the error signal is zero, we want the system to remain steady (with null derivative), when the output is less than the set-point we want the system to increase the output (positive derivative) and when the output value is greater than the set-point we want the output to decrease. The system's output derivative as seen in equation (3):

(3) $$\frac{dy}{dt}_{system} = K_1(y^* - y) + K_2\int(y^* - y)dt$$

Where $\mathbf{K_1}$ and $\mathbf{K_2}$ are diagonal matrices that can be made to vary with time, y^* – setpoint. The optimal control *u(t)* satisfies the follow conditions:

$|u(t)| \leq \alpha$,

$$\int_0^{tf} [h(x,u,d,t)^T W h(x,u,d,t)]dt = \min$$

Where W is a positive weighting matrix,

$$h(x,u,d,t) = \frac{dy}{dt} - \frac{dy}{dt}_{system}$$

$$h(x,u,d,t) = \frac{\partial g}{\partial x} f(x,u,d,t) - K_1(y^* - y) - K_2 \int (y^* - y)dt$$

System identification

Several experiments were carried out in the bioreactor cultivation of *L. linearifolium*, the data sets obtained from online and offline measurements were used to identify the parameters of a model with the structure of equations (Stephanopoulos, San 1984, Lewis 1986, Chen 1994, Krstic 1995) using also the Monod equation (8) for the kinetics of the growth rate. The method used for identification was non-linear least squares.

(4) $$\frac{dx_b}{dt} = \mu x_b - \frac{F}{W} x_b$$

(5) $$\frac{ds}{dt} = -\frac{\mu x_b}{Y_{x/s}} + \frac{F}{W}(S_F - S)$$

(6) $$\frac{dW}{dt} = F$$

(7) $$OUR = \alpha \mu x_b W + \beta x_b W$$

(8) $$\mu = \mu_{\max} \frac{s}{K_s + s}$$

Where:

α [g/g] -yield biomass/oxygen, β [g/g/d] - maintenance term for oxygen, s_F [g/l] - substrate concentration in feed, μ_{max} [l/d] - maximum growth rate, K_S [g/l] - saturation constant, $Y_{x/s}$ [g/g] - yield substrate/biomass, μ [1/d] - specific biomass growth rate, S [g/l] - substrate concentration, x_b [g/l] - biomass concentration, F [kg/d] - feeding rate, W [kg] - bioreactor weight, OUR [g/d] - oxygen uptake rate.

Observing the bioreactor

For better control and optimization of cultivation processes of *L. linearifolium* cells it is essential to know on-line these physiological parameters. Measuring is difficult, if not impossible, for many of these parameters. Therefore, any control or optimization based on physiological parameters cannot be implemented unless values can be measured on-line to provide the necessary information required by the controller. In vitro cell cultures of *Linum* species are characterised by complex, non-linear relationships involving poorly identified parameters. This makes them likely candidates for applying adaptive algorithms. For instance, to avoid explicitly modeling the specific rates, these can be treated as parameters, and estimated along with the state variables. The dynamics of the latter can then be described by simple mass-balance equations. Stephanopoulos and San (1984) followed this approach by implementing an adaptive Extended Kalman Filter (EKF). Specific rates and state variables were both satisfactorily estimated under steady-state or transient operation from measurements provided by the analysis of cultivation. The linear Kalman filtering algorithm, proposed by us, has two steps, respectively the time update and the measurement update. In the time update a one sample ahead prediction is made for the state and output variables (respectively x_{k1}, y_{k1}) and the prediction variance of the states (P_{k1}). The actual values of the input variables (u_k) and the available estimated results of the Kalman filter ($\hat{x}_k, \hat{P}_k$) at sample moment *k* are used for this prediction.

The next step is the measurement update, which takes place as soon as new data becomes available and where the prediction of the output variable (y_{k1}) is corrected. The correction is proportional to the innovation, which is the difference between the measured process output (y_{mk_1}) and the predicted output (y_{k1}). The magnitude of the correction, called the *Kalman gain* K_k, varies for successive samples and aims to minimize the error covariance ($\hat{P}_k$) for the state and output variables. Now, the measurement update gives the best estimate ($\hat{x}_{k+1}$) of the states and its variance ($\hat{P}_{k+1}$). The values for the state estimate ($\hat{x}_{k+1}$) are used as the software output. The entire set of these equations comprises what is called the discrete time Kalman filter (Lewis 1986, Chen 1994). The Kalman filter makes the prediction of the states in such way that the variance of the prediction error is minimized (Fig.1).

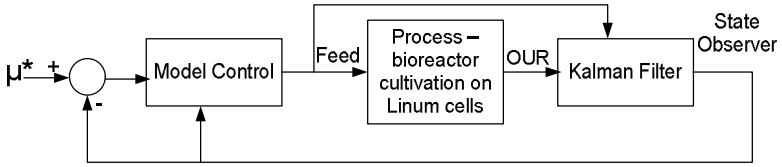

Fig.1 Block diagram of the control algorithm

Integral forwardstepping control

The most popular control strategy for existing bioreactor control is based on a nested PI controller design (Krstic 1995, Tan and Chang 1999, Taylor 1994). In this approach, PI controllers are used in both the velocity control loops and oxygen uptake. In many control applications, the nested PI controller works well and presents certain acceptable performance. However, when the system parameter uncertainties and mismatch become significant due to, for example, large variations of the bioreactor parameters and disturbances, it is difficult to achieve satisfactory performance based on the classical nested PI scheme. The nested PI controller strategy does not consider the cross-relation between the outer and inner control loops, which essentially limits its performance. In our work, a practical integral action is included in the design for this multiple loop system. This is necessary to compensate for the disturbance and eliminate the steady state error that could be produced by inaccuracy in the component and system modeling. The forward stepping algorithm presents very good response as well as rejection to disturbance. The proposed new approach can be used to replace the existing nested PI to obtain superior performance for the bioreactor velocity control loops. We was formulate control problem from the control design perspective and introduce the basic design of integrator controller. This is followed by the analysis of the system stability and asymptotic response performance. The effectiveness of the proposed design method is demonstrated through computer control of bioreactor BIOSTAT PLUS by the cultivation of suspension cultures of *L. linearifolium,* producing anticancer lignans (Tabl. 1).

Optimal control algorithm

The system was represented by differential equations instead of transfer functions. The standard model of bioreactor cultivation was:

$$\frac{dx}{dt} = Ax + Bu + v \tag{9}$$

$$y = Cx + e$$

where u is the input, y the output and x is the state. The uncertainty is represented by the disturbances v and e and by variations in the elements of the matrices A, B and C .The disturbances v and e were typically described as stochastic processes. Since the equations are linear with stochastic disturbances and the criterion is quadratic the problem was called the linear quadratic Gaussian control problem (LQG). The solution to the control problem is given by

$$u = L(x_m - \hat{x}) + u_{ff}$$

(10)

$$\frac{d\hat{x}}{dt} = A\hat{x} + Bu + K(y - C\hat{x})$$

This control law has a very nice interpretation as feedback from the error $x_m - \hat{x}$ which is the difference between the ideal states x_m and the estimated states $\hat{x}$, L is gain. The estimated states are given by the Kalman filter. In Figure 2 we show a block diagram of the controller obtained from LQG theory. In the figure we have also used a system configuration with two degrees of freedom. The system has a very attractive structure. The observer or the Kalman filter delivers an estimate of the state based on a model of the system and the input and output signals of the system.

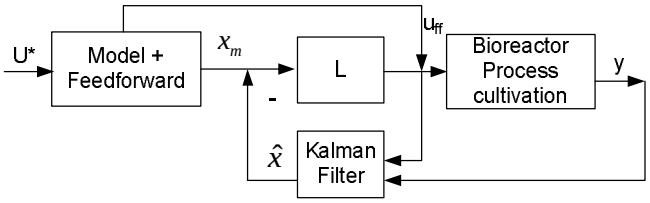

Fig. 2 Block diagram of a system state feedback

We investigated the use of 3 different strategies to control the cell growth and podophyllotoxin production in 2-L bioreactor. All of them have been shown to be applicable for cultivation of *L. linearifolium* cells. The results of a comparative study of the different control methods in 2-L stirred tank bioreactor are presented in Table 1. An agitation speed of 140 rpm was found to be sufficient to mix the culture broth in the bioreactor without causing any significant cell damage. The cultivation of *L. linearifolium* in a 2 L stirred tank bioreactor, using Model Control Method (MCM) resulted in biomass accumulation level of 25.7±0.08 g/L (DCW basis) and podophyllotoxin production of 8.64±0.12 mg/L, which represented a 60% increase in compared to that of shake flask cultivation.

Table 1. Biomass formation and podophyllotoxin production in *L. linearifolium* cells by cultivation in 2-L stirred tank bioreactor using different control methods.

Control methods	**Biomass formation** (g/L) (DCW basis)	**Podophyllotoxin production (**mg/L)
Model Control Method (MCM)	25.7±0.08	8.64 ±0.12
Integral forwardstepping control	23.67±0.11	6.32±0.05
Optimal control algorithm (LQG)	27.04±0.07	4.81±0.03

n=3; value are mean ± SD

All the controller strategies showed good performance in maintaining the set point and give better disturbance rejection for controlling bioprocess system. But MCM on batch fermentation processes using a non-linear model in the controller show good relation in maximum biomass and podophyllotoxin accumulation. They operated the process with combination of operating regimes. Moreover, MCM can be use as a model to predict the process output at future time instants. A kinetic model was used to predict culture growth and secondary metabolism in *L. linearifolium* plant cell culture. The model can be used to develop an operating strategy and control scheme for maximizing the production of plant secondary metabolites.

The results showed that the operating-regime-based modeling framework can be used as a means for modeling processes that operate over a wide range of operating conditions. The optimization made it possible to establish the optimal conditions for the biosynthesis of podophyllotoxin by *L. linearifolium*: dissolved oxygen, 50% of air saturation; agitation, 140 rpm; pH 5.6 and temperature,

26 °C, where maximal yield of 8.64 ±0.12 mg/L of podophyllotoxin was achieved - 1.6 times higher compared with the shake-flasks cultivation (Ionkova et al. 2010). Then the MCM can be used to improve performance of optimal control system in plant cell culture for production of important anticancer ariltetraline lignans.

Conclusion

To meet the growing demand by pharmaceutical industries of these anticancer compounds, three alternative strategies have been proposed in our study in order to improve the yield in batch cultivation of in vitro cultures of *L. linearifolium* - an endemic rare plant species, which accumulate predominant podophyllotoxin. The evaluation of these strategies shows that these methods can successfully improve the cell yield of the bioreactor. All the controller strategies showed good performance, but the best one for the production of podophyllotoxin in *L. linearifolium* in the pilot bioreactor was found to be Model Control Method. This is one method for improving the productivity and, hence, cost of a process.

ACKNOWLEDGEMENTS

Financial support from Ministry of Education and Science, Sofia, Bulgaria (D002-128/2008 and DTK 02-1/2009) is acknowledged.

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