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ORIGINAL PAPER

Optimisation of Synthetic Medium Composition for Levorin Biosynthesis by *Streptomyces levoris* 99/23 and Investigation of its Accumulation Dynamics Using Mathematical Modelling Methods

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Abstract

The composition of a synthetic culture medium for levorin biosynthesis by *Streptomyces levoris* 99/23 was optimised using mathematical modelling methods. The optimal concentrations of the medium components were established by means of an optimum composition design at three factor variation levels. An adequate regression model was obtained. Levorin biosynthesis by *Streptomyces levoris* 99/23 in the optimised synthetic medium was over 38% higher than in the initial medium. The antibiotic biosynthesis dynamics in the optimised culture medium was studied by means of a non-linear differential equation system. The resultant model was valid.

Key words: *Streptomyces levoris* 99/23, biosynthesis dynamics, mathematical modelling, optimisation of levorin biosynthesis

Introduction

Levorin is an antifungal preparation widely used in medicine. The antibiotic biosynthesis mechanism and the characteristics of its producers in a physiological aspect are studied mainly by means of synthetic culture media (Belousova *et al.*, 1970; Jakovleva, 1980). These media have strain-specific compositions which are determined experimentally. Culture medium optimisation in a quantitative and qualitative aspect using mathematical modelling methods is insufficiently studied. Orthogonal Latin rectangles were used by Jakovleva (1980), and linear models were applied by Gotchev *et al.* (2002). There are individual reports on biosynthesis description using neural networks (Xian-Fa *et al.*, 2000). From a practical point of view, however, they are more suitable for process control rather than investigation since kinetic constant values are most often hidden either in the architecture or in the weight coefficients of the neural network.

This paper aimed to determine the optimal concentrations of the synthetic culture medium components for levorin biosynthesis by *Streptomyces levoris* 99/23 and study levorin accumulation dynamics using mathematical modelling methods.

Experimental

Materials and Methods

Microorganism. A *Streptomyces levoris* 99/23 strain stored in a lyophilised form in the Biotechnology Department's collection at UFT was used as a levorin producer (Kozhuharova *et al.*, 2002). The culture was maintained on a medium described by Kozhuharova *et al.* (2008).

Media and cultivation conditions. The initial nutrient medium for *S. levoris* 99/23 cultivation, which was subject to optimization, had the following composition (%): glucose 1.5; starch 2; $(NH_4)_2SO_4$ 0.6; KH_2PO_4 0.005; KCl 0.1; $MgSO_4$ 0.25; $CaCO_3$ 0.3. After pH adjustment to 7.2, and sterilization at 121°C for 30 min, the nutrient medium was inoculated with

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2% (v/v) spore inoculum containing 2.10^9 cfu/ml. Strain cultivation and levorin biosynthesis were carried out in 500 ml Erlenmayer flasks containing 50 ml of each nutrient medium at a temperature of 28°C, on a rotary shaker (220 min^{-1}) for 96 h.

Mathematical modelling. The optimal concentrations of medium components were determined using optimal composition design with three variation levels of the factors (Koleva *et al.*, 2005; Mason *et al.*, 2003). Such an approach enables generation of non-linear regression models with a minimum number of experiments:

$$Y_{mod} = b_0 + \sum_{i=1}^{k} b_i.x_i + \sum_{i=1}^{k} b_{ii}.x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} b_{ij}.x_j.x_j \qquad (1)$$

where: Y_{mod} is the predicted response, b_i, b_{ij} and b_{ii} are coefficients accounting for the effect of each factor (x_i), of their interrelations ($x_i.x_j$), and those to the square of two (x_i^2) respectively, and k is the number of factors.

The experimental data statistical processing and the results analysis were performed using Anova (Microsoft Excel 2003).

The process dynamics was studied by submerged cultivation of the strain in the optimised culture medium. The input parameter values were approximated by third order spline functions (Mathews and Fink, 2001). The calculation and optimisation procedures were performed within the Eureka software environment (The Software Eureka 2000). The graphic presentation was based on Microsoft Excel 2003 and Sigma Plot 9.0.

Assays. Levorin concentration in the culture medium was analysed according to the spectrophotometric method suggested by Bob *et al.* (1978) and expressed in mg/ml. One antibiotic activity unit (IU) corresponds to 0.04 μg of levorin (Bolshakova *et al.*, 1989). Reducing sugars (substrate) concentration was determined by the dinitrosalicylic acid method (Miller, 1959). The biomass quantity was determined after drying at 105°C to constant weight. pH was measured potentiometrically.

Results and Discussion

Following a series of single-factor experiments, the variation interval for the basic culture medium components was determined. The real and coded values of the independent variables are shown in Table I. The remaining constituents were fixed at the following levels (%): KCl 0.1; $MgSO_4$ 0.25; $CaCO_3$ 0.3.

The experimental data was formed as the mean value of the results of six parallel experiments. The design matrix, the experimental results (Y_{exp}) and model values (Y_{mod}) obtained by equation (2) are presented in Table II.

Table I
Real and coded values of independent variables

Factor (%)	Coded value		
	–1	0	+1
X_1: glucose	1.0	1.5	2.0
X_2: starch	1.0	2.0	3.0
X_3: $(NH_4)_2SO_4$	0.4	0.6	0.8
X_4: KH_2PO_4	0.001	0.0055	0.01

Table II
Optimum composition design for 4 factors and three levels of their variation

No	X_1	X_2	X_3	X_4	Y_{exp} (mg/ml)	Y_{mod} (mg/ml)
1	1	1	1	1	0.667	0.597
2	1	1	1	–1	0.724	0.748
3	1	1	–1	1	0.785	0.737
4	1	1	–1	–1	0.576	0.603
5	1	–1	1	1	1.242	1.135
6	1	–1	1	–1	0.581	0.581
7	1	–1	–1	1	0.860	0.806
8	1	–1	–1	–1	0.529	0.537
9	–1	1	1	1	0.718	0.781
10	–1	1	1	–1	0.726	0.751
11	–1	1	–1	1	0.851	0.921
12	–1	1	–1	–1	0.573	0.605
13	–1	–1	1	1	0.894	0.950
14	–1	–1	1	–1	0.572	0.578
15	–1	–1	–1	1	0.588	0.622
16	–1	–1	–1	–1	0.527	0.535
17	–1	0	0	0	0.592	0.693
18	1	0	0	0	0.691	0.693
19	0	–1	0	0	0.903	0.820
20	0	1	0	0	0.812	0.820
21	0	0	–1	0	0.714	0.739
22	0	0	1	0	0.700	0.646
23	0	0	0	–1	0.635	0.691
24	0	0	0	1	0.620	0.590

The analytical expression of the regression equation obtained is:

$$Y_{mod} = 0.6928 + 0.0472.X_3 + 0.1007.X_4 - 0.0467.X_1.X_2 - 0.0459.X_2.X_3 - 0.0595.X_2.X_4 - 0.0454.X_1.X_2.X_4 - 0.0714.X_2.X_3.X_4 + 0.1276.X_2^2 - 0.1024.X_4^2 \qquad (2)$$

The model was adequate at confidence level $\alpha = 0.05$ and degrees of freedom $\nu = 9$ (Table III).

The analysis of (2) revealed several considerations.

The two carbon sources (X_1, X_2) were not present on their own in the model but the coefficient before X_2^2 in (2) had higher positive value. Its effect in Y_{mod} in relation to b_0 was 18.4%. This showed that *S. levoris 99/23* preferred a carbon source with a relatively high molecular mass. On the other hand, X_1

Table III
Statistical analysis results according to Anova

Parameter	Df	SS	MS	F	Significance F
Regression	9	0.5176539	0.057517098	10.21185	9.32311E-05
Residual	14	0.0788534	0.005632389		
Total	23	0.5965073			

Df – degree of freedom; SS – sum square; MS – mean square; F – Fisher coefficient

participated in two terms of (2) with total weight of 13.3% in relation to b_0. Since the effect of both factors in Y_{mod} is commensurate, there is sufficient ground to believe that they are in optimum correlation ensuring the absolute extremum of (2).

X_4 had a pronounced individual influence in the model (15%, which is comparable to that of X_2^2). KH_2PO_4 was a source of phosphorus, an important element for the *S. levoris* growth and levorin biosynthesis regulator. In this respect, the results obtained are in agreement with the theoretical formulations and data reported by other authors (Belousova *et al.*, 1970).

The nitrogen source can be seen as having no significant effect on levorin biosynthesis.

The conditions maximising (2) were found by means of a gradient optimisation method (The Software Eureka 2000):

$$Y_{mod}^{max} = 1.135 \text{ mg/ml, at: } X_1 = +1;\ X_2 = -1;\ X_3 = +1;\ X_4 = +1 \quad (3)$$

The response function (2), in graphic form, with variation of X_1 and X_2 within the limits set in Table I and optimum values of X_3 and X_4 (+1), is presented on Fig. 1.

The hypothesis of equality of the mathematical expectation of the experiment results Y_{exp}^{max} under the optimal conditions with that of the predicted $Y_{mod}^{max} = 1.135$ was checked (Mason *et al.*, 2003). The values (mg/ml) were as follows: $Y_{exp,1}^{max} = 1.20$; $Y_{exp,2}^{max} = 1.22$; $Y_{exp,3}^{max} = 1.16$; $Y_{exp,4}^{max} = 1.15$; $Y_{exp,5}^{max} = 1.10$; $Y_{exp,6}^{max} = 1.12$; $Y_{exp,7}^{max} = 1.06$; $Y_{exp,8}^{max} = 1.00$. For degrees of freedom $\nu = 7$ and confidence level $\alpha = 0.05$, $t_{crit.} = 2.365$ (Student's table). Since $t_{calc.} = 0.535 < t_{crit.}$, there was no statistically significant difference between Y_{exp}^{max} and Y_{mod}^{max}

A significant increase in levorin biosynthesis was detected with the optimised medium. The yield achieved was with 38% higher in comparison to the yield with the initial medium.

The dynamics of levorin biosynthesis by *S. levoris* 99/23 with the optimised medium was modelled by means of a system of the following non-linear differential equations:

$$\frac{dX(t)}{dt} = \mu(t)\, X(t) \quad (4)$$

$$\frac{dS(t)}{dt} = -\frac{1}{Y_1}\frac{dX(t)}{dt} - Y_2 X(t) \quad (5)$$

$$\frac{dP(t)}{dt} = \alpha_1 \frac{dX(t)}{dt} + \alpha_2 X(t) \quad (6)$$

$$\mu(t) = \mu_m \frac{S(t)}{k_s + S(t)} - \beta X(t) \quad (7)$$

$X(t)$, $S(t)$, and $P(t)$ are the biomass concentrations, substrate concentration, and levorin concentration respectively when the process operates in the periodic mode, Y_1 is an economic coefficient, Y_2 is related to the rate of substrate assimilation by the cells in a stationary phase of the process, k_s is a saturation constant, μ_m is the maximum specific growth rate, β is the decay coefficient, α_1 is the coefficient of substrate transformation into a metabolism product, and α_2 is levorin accumulation rate in the stationary phase of the process.

Using (7), the microbial population growth was modelled in the presence of substrate limitation – modified Verhulst law.

The experimental data were formed by using the mean value of the results from six parallel experiments on the process dynamics. The lag phase time (24 h) was excluded from the data set.

The kinetic constant values in the model were determined using an optimisation procedure minimising the following criterion:

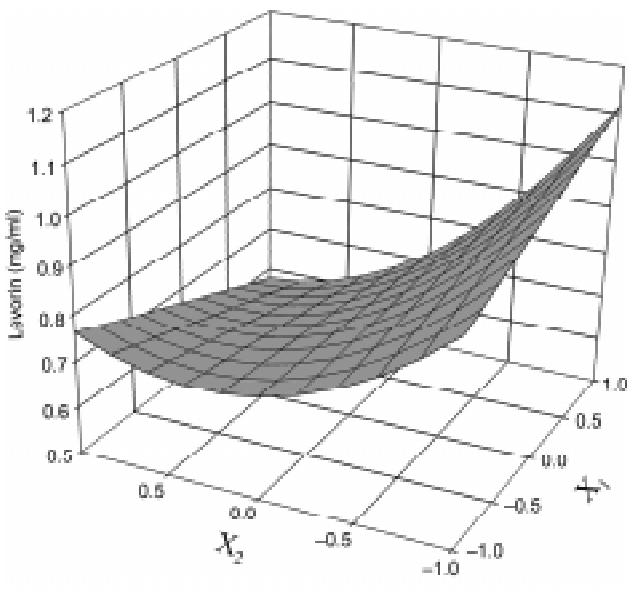

Fig. 1. $Y_{mod} = f(X_1, X_2)$ at an optimum value of X_3 and $X_4 + 1$.

$$J = \sum_{i=1}^{n} [(X_{\text{exp},\,i} - X_{\text{mod},\,i})^2 + (S_{\text{exp},\,i} - S_{\text{mod},\,i})^2 + (\mathrm{P}_{\text{exp},\,i} - \mathrm{P}_{\text{mod},\,i})^2] \rightarrow \min \quad (8)$$

where exp, *i* and mod, *i* denote the process parameters according to the experimental data and models (4–7), and *n* is the number of observations.

With this setup, the numerical values of the kinetic constants for the model were determined to be as follows:

$\mu_m = 0.05583\ \text{h}^{-1}$; $k_s = 0.97$ mg/ml;
$\beta = 0.01054$ ml/mg.h; $Y_1 = 0.233$;
$Y_2 = 0.0077\ \text{h}^{-1}$; $\alpha_1 = 0.169$; $\alpha_2 = 0.00117\ \text{h}^{-1}$

The process dynamics is presented graphically in Fig. 2, Fig. 3 and Fig. 4. There was a good coincidence between the experimental and model results. $P(t)$ reached a maximum equal to 1.08 mg/ml at t=131.5 h (Fig. 4). This value was obtained after approximation of the analytically calculated $P(t)$ data according to (6), with a second-order spline function and maximisation of $P(t)$, within the 108–144 h time interval and degree of freedom *t*. No check of the experiment reproducibility was run since it generally coincided with that of the studies on the process statics.

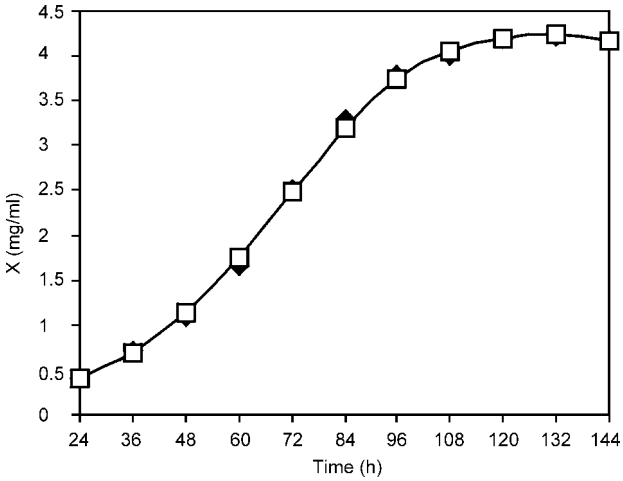

Fig. 2. Growth dynamics of *S. levoris* 99/23: (◆) experimental data; (□) model data.

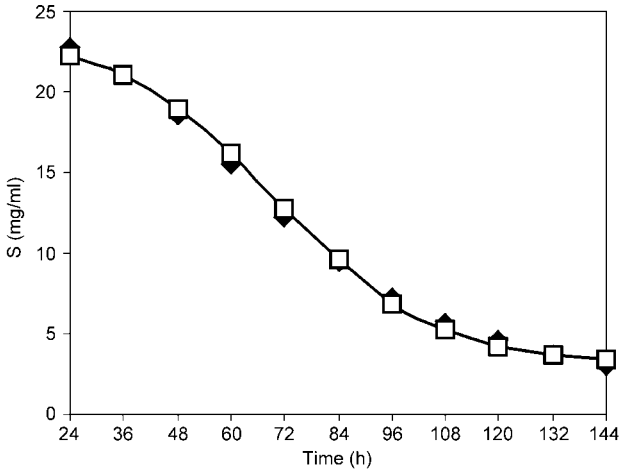

Fig. 3. Dynamics of substrate assimilation by *S. levoris* 99/23: (◆) experimental data; (□) model data

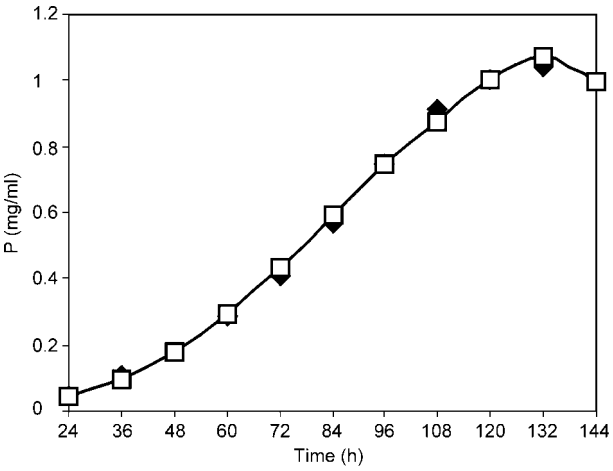

Fig. 4. Dynamics of levorin biosynthesis by *S. levoris* 99/23: (◆) experimental data; (□) model data.

The kinetic constants can be interpreted in the following manner in a biotechnological aspect.

Under the experimental conditions, the value of decay coefficient β, was 5.3 fold lower than the value of μ_m. At time point t = 131.5 h, the available biomass exceeded the initial biomass by 10.7 folds.

Over 23% of the substrate was assimilated for biomass accumulation (Y_1), and around 18.5% was used for maintaining the life activity in the stationary phase of the process. Since the values mentioned were close, it could be considered that approximately the same substrate amount was spent for both purposes.

The main antibiotic quantity was synthesised in the process stationary phase, and it was 6 fold higher than the value in the exponential phase, at a priori specified stationary phase duration of 36 h.

The ratio $\alpha_1/(\alpha_2.\Delta t)$ for Δt = 36 h, in the stationary phase was 1:6. This came as another proof that levorin biosynthesis took place mainly at this stage of the microbial population growth.

At the end of the process, over 15.6% of the substrate remained unassimilated (Fig. 3). This was an indication for the presence of a critical value in respect to S_0 at which, the function would reach its maximum along with the complete substrate utilisation. Such an effect was not registered under the conditions of the experiment.

The material balance of the process came to the following considerations. The substrate consumption for biomass accumulation in the exponential growth phase of *S. levoris* 99/23 was about 23.3%, and the value for life activity during the process stationary phase was about 18.5%. About 15.6% of the substrate was unassimilated at the end of the biotechnological process. For antibiotic synthesis in the exponential growth phase about 16.9% of the substrate was used, and the corresponding value for the stationary phase

was 27.7%. Total carbon source consumption was determined to be 102%.

We consider that the results obtained provide an objective idea of the material balance in the system taking into account the subjective, methodological and instrumental error in the experimental data analysis.

The dynamics model of the levorin biosynthesis by *S. levoris* 99/23 was valid. It described in detail even the lysis processes at the end of the stationary phase. In proof of this statement, the experiment-model error dispersion values are presented for all observation points as follows: σ^2_x=0.0025; σ^2_s=0.26; σ^2_p=0.0005. The main share in criterion (8) is mainly attributed to *S* since its natural values are 100 and more times higher than those of *X* and *P*.

Conclusion. As a result of the optimisation of the nutrient medium for levorin biosynthesis by *S. levoris* 99/23 using mathematical modelling methods, the optimal composition of the medium was determined. The yield achieved with this medium was 38% higher in comparison to the initial one.

The dynamics of the antibiotic biosynthesis by *S. levoris* 99/23 was studied by means of a non-linear differential equation system. The kinetic constant values were calculated for Verhulst model, describing the presence of substrate limitation. A valid model of the dynamics of levorin biosynthesis by *S. levoris* 99/23 was obtained and the material balance of the process was assayed.

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