Modelling and Optimization of the (R)-(+)-3,4-dihydroxyphenyllactic Acid Production Catalyzed with D-lactate Dehydrogenase from *Lactobacillus leishmannii* Using Genetic Algorithm

Z. Findrik, M. Poljanac,* and Đ. Vasić-Rački[+]

Faculty of Chemical Engineering and Technology, University of Zagreb,
Savska c. 16, HR-10000 Zagreb, Croatia
*Ekonerg – Energy and Environmental Protection Institute,
Koranska 5, HR-10000 Zagreb, Croatia

A mathematical model for the enzymatic kinetics of the synthesis of (R)–(+)–3,4-dihydroxyphenyllactic acid (DHPL) was developed. The synthesis was catalyzed by D-lactate dehydrogenase from *Lactobacillus leishmannii*. Since this enzyme requires NADH as a coenzyme, formate dehydrogenase system was used for NADH regeneration. Kinetic constants of both enzymes were estimated independently from initial reaction rate experiments. The developed mathematical model was verified by the batch reactor experiment (volumetric productivity in this experiment was 4.76 g dm^{-3} d^{-1}). Optimization of initial reaction conditions for DHPL synthesis was performed using the genetic algorithm (GA). The genetic algorithm as a flexible optimization tool had been used to obtain the experimental conditions where maximal volumetric productivity could be achieved. The optimal initial conditions were found in the investigated parameter area: $c_{\text{3,4-dihydroxyphenylpyruvic acid}}$ = 4.69 mmol dm^{-3}, $c_{\text{NAD+}}$ = 4.95 mmol dm^{-3}, c_{formate} = 36.85 mmol dm^{-3}, $\gamma_{\text{D-lactate dehydrogenase}}$ = 3 mg cm^{-3}, $\gamma_{\text{formate dehydrogenase}}$ = 2.94 mg cm^{-3} and the reaction time 8.5 min. At these conditions volumetric productivity of 93.06 g dm^{-3} d^{-1} can be achieved.

Keywords:
Enzymatic synthesis, mathematical modelling, optimization, genetic algorithm

Introduction

The optimization of bioprocesses is very important for their efficiency,[1] as well as for economic reasons. Among all the recent nonlinear optimization techniques, the genetic algorithm is the most studied and general one. The major advantage of genetic algorithms over other conventional optimization techniques is the flexibility it provides in framing the objective function and constraints.[2] It can be used in cases where interaction between higher number of parameters is present, which usually narrows the choice of optimization methods.[3] Beside genetic algorithm as a stochastic optimization method, statistical methods represent the alternative. Experimental design as a method has a disadvantage because of its assumption that no interactions occur between parameters of the considered parameter space. Factorial experimental design is based on the assumption that the interaction between parameters and target function can be described by the first order approach which in most cases permits interactions between two parameters. The restriction of statistical methods in the search of optimum is also in the fact that the target function must be unimodal, otherwise a local optimum can be identified instead of the global optimum (because of the random choice of parameter range).[4]

(R)–(+)–3,4-dihydroxyphenyllactic acid (DHPL), also known as Danshensu, is an interesting pharmacological compound that is used for the treatment of menstrual disorder, menostasis, menorrhalgia, insomnia, blood circulation diseases, and angina pectoris.[5] It can be found in *Salvia miltiorrhiza*, also known as Danshen, which is listed in the Chinese Pharmacopoeia, and is a traditional Chinese medicine. It was synthesized enzymatically earlier for the purpose of rosmarinic acid synthesis[6] and traditionally it is being extracted from the red coloured root of the Cinnabar Sage, *Salvia miltiorrhiza*. The procedure for its chemical synthesis is also known, but racemic mixture is the product of such synthesis, as well as various by-products that make the separation more difficult.

Dehydrogenases are a large class of enzymes that require presence of coenzyme for their activity. Since D-lactate dehydrogenase from *Lactobacillus*

[+] Corresponding author: Faculty of Chemical Engineering and Technology, University of Zagreb, Savska c. 16, HR-10000 Zagreb, Croatia,
Phone: + 385 1 4597 104, Fax: + 385 1 4597 133,
E-mail: dvracki@marie.fkit.hr

leishmannii acquires NAD^+/NADH for its activity and, both, NAD^+ and NADH are quite expensive, it is of great concern to induce the coenzyme regeneration systems to lower coenzyme consumption. Formate dehydrogenase (FDH) can be used as one of the solutions. The advantage of this approach is to shift the equilibrium of the desired product-forming reaction to a complete conversion, due to the irreversibility of the oxidation of formate to CO_2 and its easy removal as a gaseous product.[7] It was developed more than 14 years ago in a process with continuous cofactor regeneration using isolated enzymes for the production of L-*tert*-leucine.[8]

The objective of the present work was mathematical model-based optimization of the DHPL enzymatic synthesis by the use of genetic and simulation (Episode) algorithm. DHPL enzymatic synthesis was catalyzed by D-lactate dehydrogenase from *Lactobacillus leishmannii*, and the NADH was regenerated by formate dehydrogenase system. The reaction scheme is presented in Fig. 1. There are six parameters that influence the outcome of this reaction (the concentrations of 3,4-dihydroxyphenylpyruvate, formate, NAD^+, D-lactate dehydrogenase and formate dehydrogenase and the reaction time). Since they all depend one on another, the use of genetic algorithm as a stochastic optimization method seemed to be the best choice for the optimization.

Materials and methods

Materials

NAD^+, NADH, 3,4-dihydroxyphenyl-L-alanine (L-DOPA), L-amino acid oxidase from snake venom *Crotalus adamanteus* (E.C. 1.4.3.2.), beef liver catalase (E.C. 1.11.1.6.), and formate dehydrogenase from *Candida boidinii* (E.C. 1.2.1.2.) were purchased from Fluka (Germany). D-lactate dehydrogenase from *Lactobacillus leishmannii* (E.C. 1.1.1.28.) was purchased from Sigma (Germany). Na_2HPO_4 and K_2HPO_4 were purchased from Kemika (Croatia).

Methods

D-lactate dehydrogenase activity assay

The activity of *Lactobacillus leishmannii* D-LDH was measured according to the standard enzyme assay.[9] The standard assay mixture contains the solution of substrate DHPP, NADH solution, D-LDH solution and phosphate buffer (0.2 mol dm^{-3}, pH 7.6). The change of NADH absorbance at 340 nm was followed on the spectrophotometer (Shimadzu, UV 1601) and activities were calculated by using a molar extinction coefficient of 6.220 cm^2 μmol^{-1}. All measurements were carried out in quartz cuvettes in a total volume of 1 cm^3. The cuvettes were thermostated and the activity was measured at 25 °C.

Formate dehydrogenase activity assay

Formate dehydrogenase activity was measured according to the principal described above. The assay contained ammonium formate solution, NAD^+ and FDH solution, as well as the phosphate buffer (0.2 mol dm^{-3}, pH 7.6). The change in NADH absorbance was followed spectrophotometrically as in D-LDH assay.

HPLC analysis

Concentration of substrate DHPP and product DHPL were followed by HPLC (Sykam) with re-
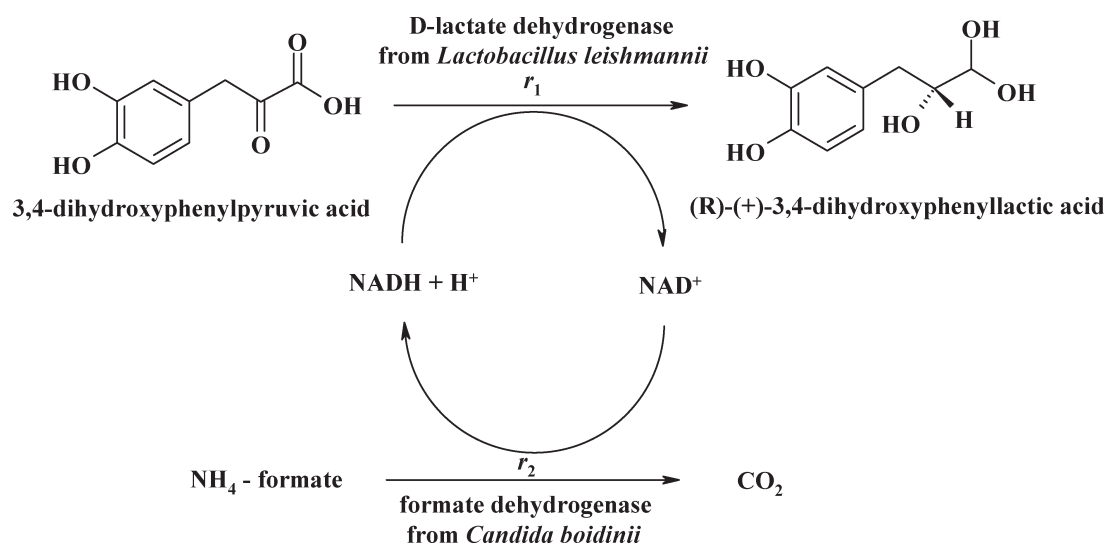

F i g . 1 – *Reaction scheme of the D-lactate dehydrogenase catalyzed DHPL synthesis coupled by NADH regeneration system catalyzed by formate dehydrogenase*

verse phase Varian column for organic acids (5 μm) and UV detection at 280 nm. The mobile phase was octilamine and acetonitrile at the ratio: 800 : 200 (v/v).The flow-rate was 1 cm³ min⁻¹ and the analysis was carried out at 40 °C. Retention times of DHPP and DHPL were 4.81 and 3.86 min, respectively.

Conversion experiments

Experiments were carried out in the batch reactor which was thermostated at 25 °C.

DHPP synthesis

DHPP was prepared enzymatically using L-DOPA (3,4-dihydroxyphenyl-L-alanine) as a substrate. The enzyme that catalyzed the reaction of oxidative deamination of L-DOPA was L-amino acid oxidase from snake venom *Crotalus adamanteus*. Catalase from beef liver was also added in the reaction system to prevent oxidative decarboxylation of DHPP. The concentration of L-DOPA was 5 mmol dm⁻³, the concentration of L-amino acid oxidase was 0.61 mg cm⁻³, and the concentration of catalase was 0.1 mg cm⁻³. The reaction was carried out in the phosphate buffer at pH 7.8. The reactor volume was 10 cm³.

DHPL synthesis

The DHPL synthesis was carried out at the following initial conditions: c_{DHPP} = 3.00 mmol dm⁻³, c_F = 19.55 mmol dm⁻³, c_{NAD^+} = 28.35 mmol dm⁻³, t = 180 min, $\gamma_{D\text{-}LDH}$ = 0.20 mg cm⁻³, γ_{FDH} = 0.67 mg cm⁻³. The reactor volume was 5 cm³.

Mathematical modelling

A complete mathematical description of the DHPL synthesis requires detailed understanding of the kinetics of both enzymes. The influence of each reaction media compound involved was determined using the enzyme assays and the initial reaction rate method. Kinetic parameters were estimated from these data. Each parameter was estimated independently from the dependence of the initial reaction rate on the substrate concentration (see results Fig. 3). These parameters were used for the development of the mathematical model, which consists of kinetic equations 1 and 2, and mass balance equations for the batch reactor 3, 4, 5, 6 and 7. Equation 1 represents D-LDH reaction kinetics, and equation 2 FDH reaction kinetics. The reverse reaction of D-LDH was neglected since it is extremely slow, and there is NADH regeneration in the system which moves the equilibrium to the side of DHPL. FDH reverse reaction was also neglected since gas-

eous CO_2 is constantly being removed from the system.

$$r_1 = \frac{V_{m1} \cdot c_{DHPP}}{K_m^{DHPP} + c_{DHPP}} \cdot \frac{c_{NADH}}{K_m^{NADH} + c_{NADH}} \quad (1)$$

$$r_2 = \frac{V_{m2} \cdot c_F}{K_m^F + c_F} \cdot \frac{c_{NAD^+}}{K_m^{NAD^+} \cdot \left(1 + \dfrac{c_{NADH}}{K_i^{NADH}}\right) + c_{NADH^+}} \quad (2)$$

It was necessary to write mass balances for the batch reactor to predict the performance of the reaction system (Eq 3–7). The mass balances were written for each component of the system: DHPP, DHPL, formate, NAD⁺ and NADH.

$$\frac{dc_{DHPP}}{dt} = -r_1 \cdot \gamma_{D\text{-}LDH} \quad (3)$$

$$\frac{dc_{DHPL}}{dt} = r_1 \cdot \gamma_{D\text{-}LDH} \quad (4)$$

$$\frac{dc_F}{dt} = -r_2 \cdot \gamma_{FDH} \quad (5)$$

$$\frac{dc_{NAD^+}}{dt} = r_1 \cdot \gamma_{D\text{-}LDH} - r_2 \cdot \gamma_{FDH} \quad (6)$$

$$\frac{dc_{NADH}}{dt} = -r_1 \cdot \gamma_{D\text{-}LDH} + r_2 \cdot \gamma_{FDH} \quad (7)$$

Data handling

Evaluation of the kinetic constants

Model parameters were estimated by non-linear regression analysis using Simplex and Least Squares method implemented in SCIENTIST software.[10] They were evaluated by fitting the model to the experimental data (enzyme kinetics – the initial rate method). Each parameter was estimated from the independent data set of approximately 10 experimental points (see results Fig. 3). The calculated data were compared with the experimental data, re-calculated in the optimization routine, and fed again to the integration step until a minimal error between experimental and integrated values was achieved. The residual was defined as the sum of squares of the differences between the experimental and calculated data.

Optimization of the reaction system in the batch reactor using genetic algorithm and simulation software

After estimation of all the kinetic constants, model for the batch reactor was simulated by SCI-

ENTIST. It was "Episode" algorithm for stiff system of differential equations, implemented in the SCIENTIST software that was used for simulations. SCIENTIST software is a program package that incorporates simplex method and least squares method for non-linear estimation of parameters. It also includes various methods of integration: Runge-Kutta, Error controlled Runge-Kutta, Bulirsch-Stoer, Episode (Adams) and Episode (Stiff). It calculates the statistics of the estimated data, which include: standard deviation of data, sum of squared deviation, R^2, coefficient of determination, coefficient of correlation, model selection criteria, standard deviation of estimated parameters

Genetic algorithm[11] (GA) was used to generate initial conditions of reactor simulations. Authors used GALOP (Genetic algorithm for the Optimization of Processes) software, program version 1.24, 1995, which was developed at the Institute of Biotechnology, Research Centre Jülich, Germany. Number of individuals in the generation was fixed at 8. The initial generation was generated at random. The probability for crossover to occur was set at 0.95 and mutation rate was set at 0.01. The best individual was always included in the next generation. Binary coding was used. The length of one individual's gene was 55 bits. Since high volumetric productivity was the target function its weight factor was set to 1.

The principle of the model based optimization was as follows: GA offers the first random population of 8 individuals with given characteristics (initial concentrations of substrates and enzymes and the time of the reaction). These values were assigned to the program written in SCIENTIST (which consists of kinetic and mass balance equations of the mathematical model – Eq 1–7) and the reactions in the batch reactor at these conditions were then simulated. Values for volumetric productivity were written in GA as return information, which GA used for further adjustments of the next generation individuals. The optimization was carried out until satisfactory standard deviation of the target function is achieved. This procedure is simplified in Fig. 2.

Results and discussion

Enzyme kinetics

Kinetic constants of the mathematical model (Eq 1 and Eq 2) were estimated from the independent experimental initial reaction rate data (Figure 3) and are presented in Table 1. It can be seen from the value of the estimated constants for D-LDH that even though the enzyme appears to be quite spe-
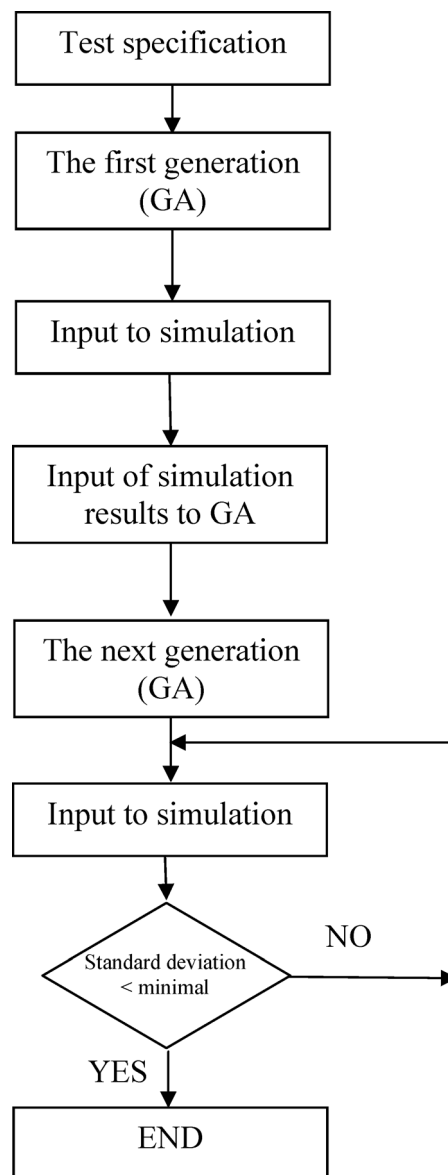

F i g . 2 – *Flowchart of the optimization procedure*

T a b l e 1 – *Kinetic parameters of the DHPP reduction by D-LDH coupled with FDH coenzyme regeneration system*

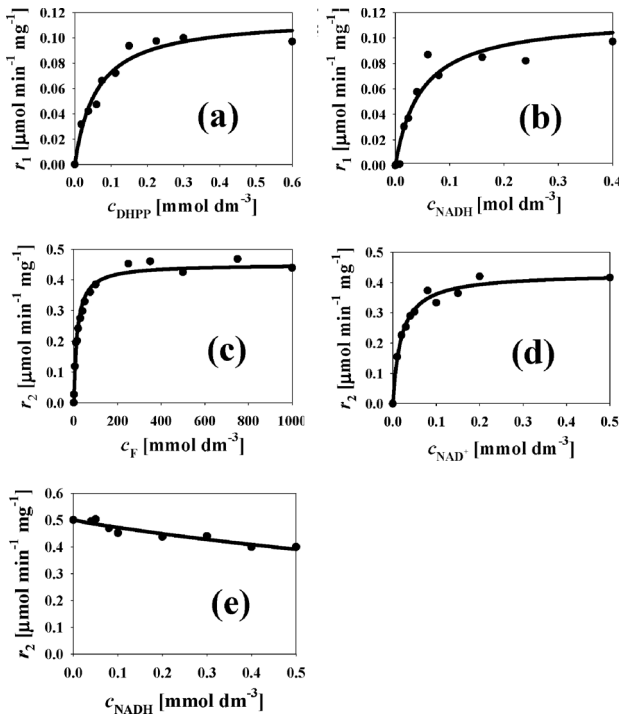| Parameter	Value	Unit
V_{m1}	0.116 ± 0.120	μmol min^{-1} mg^{-1}
V_{m2}	0.436 ± 0.014	μmol min^{-1} mg^{-1}
$K_m^{NAD^+}$	0.0192 ± 0.0023	mmol dm^{-3}
K_m^{NADH}	0.0474 ± 0.0150	mmol dm^{-3}
K_m^{DHPP}	0.0605 ± 0.0070	mmol dm^{-3}
K_m^{F}	15.6615 ± 3.3563	mmol dm^{-3}
K_i^{NADH}	0.0281 ± 0.0050	mmol dm^{-3}

F i g . 3 – *Results of enzyme kinetics investigation and estimation of kinetic constants of D-LDH : (a) the effect of DHPP concentration on the initial reaction rate (γ_{LDH} = 7.0 mg cm^{-3}, c_{NADH} = 5 mmol dm^{-3}), (b) the effect of NADH concentration on the initial reaction rate (γ_{LDH} = 7.0 mg cm^{-3}, c_{DHPP} = 3.0 mmol dm^{-3}) and FDH: (c) the effect of formate concentration on the initial reaction rate (γ_{FDH} = 8.0 mg cm^{-3}, c_{NAD^+} = 4 mmol dm^{-3}), (d) the effect of NAD$^+$ concentration on the initial reaction rate (γ_{FDH} = 8.0 mg cm^{-3}, c_F = 1 mol dm^{-3}), (e) the effect of NADH concentration on the initial reaction rate (γ_{FDH} = 8.0 mg cm^{-3}, c_F = 5.0 mol dm^{-3}, c_{NAD^+} = 4.0 mmol dm^{-3}). All experiments are carried out in the phosphate buffer (0.2 mol dm^{-3}, pH 7.6).*

cific (low K_m value) to DHPP as a substrate, its specific activity is not considerably high (0.116 μmol min^{-1} mg^{-1}). There was no inhibition present in the reaction of DHPP reduction. In the reaction of formate oxidation NADH as a reaction product inhibits the enzyme (FDH). According to this information mathematical model describing initial reaction rate was developed. D-LDH kinetics was described with double-substrate Michaelis-Menten equation, and FDH kinetics was described with double substrate Michaelis-Menten equation with competitive product inhibition by NADH (Eq 1–2).

Model validation by the batch reactor experiment

The purpose of this experiment, at the initial conditions mentioned earlier was to validate the mathematical model (Eq 3–7) since it was going to be used for optimization. Experimental results are presented in Fig. 4. The concentrations were given as non-dimensional since there is no standard available for both compounds. The volumetric produc-
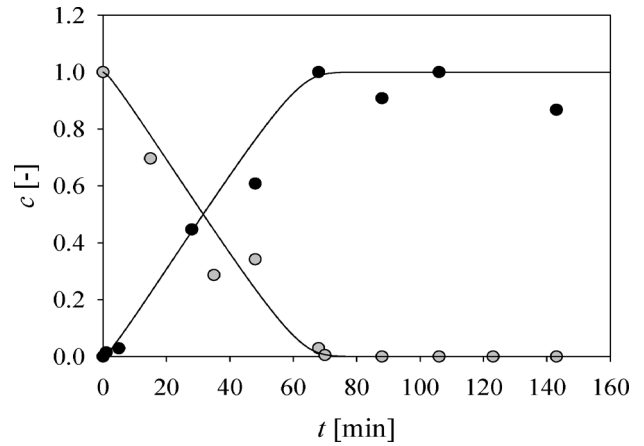

F i g . 4 – *Experiment carried out near optimal conditions, model validation (● concentration of DHPL, ○ concentration of DHPP, model simulation) (c_{DHPP} = 1.5 mmol dm^{-3}, c_F = 19.55 mmol dm^{-3}, c_{NAD^+} = 28.35 mmol dm^{-3}, γ_{D-LDH} = 0.20 mg cm^{-3}, γ_{FDH} = 0.67 mg cm^{-3}, t = 140 min).*

tivity at the end of this batch experiment was 4.76 g dm^{-3} d^{-1}. Since this experiment verified the batch reactor mathematical model, the next step was the optimization procedure.

Optimization of the reaction system

The aim of the optimization was to find initial reaction conditions at which maximum value of volume productivity (defined according to Eq 8) in the batch reactor could be achieved.

$$Q_p = \frac{c_{DHPL} \cdot M_{DHPL}}{t} \qquad (8)$$

These initial conditions include concentrations of DHPP, NAD$^+$, formate, optimal enzyme concentrations (D-lactate dehydrogenase and formate dehydrogenase), and reaction time. The parameter area in which an optimum was searched is presented in Table 2. Optimization results are pre-

T a b l e 2 – *Start values of investigated parameters, investigated area of parameters (defined by lower and upper border) and step of the model-based optimization*

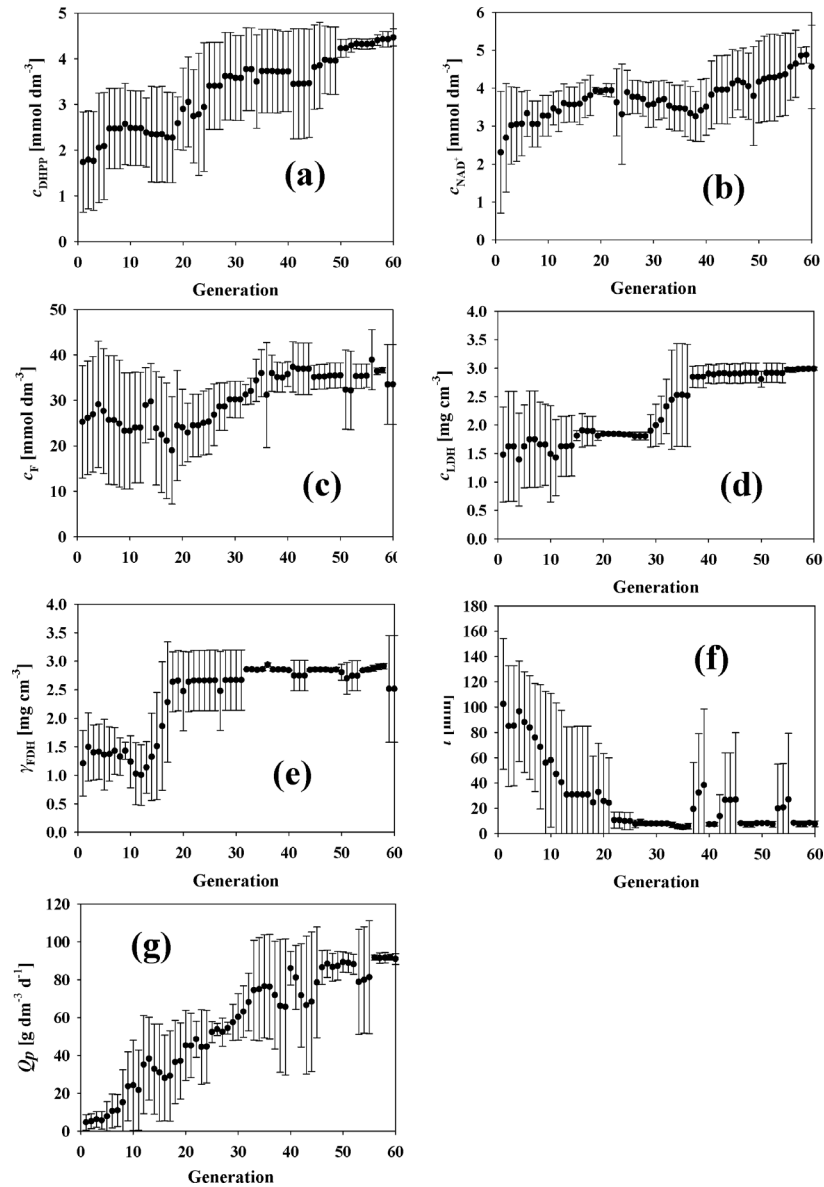| Initial conditions	Unit	Step	Lower border	Upper border
c_{DHPP}	mmol dm^{-3}	0.01	0.01	5.00
c_F	mmol dm^{-3}	0.05	0.05	50.00
c_{NAD^+}	mmol dm^{-3}	0.01	0.01	5.00
γ_{LDH}	mg cm^{-3}	0.01	0.01	3
γ_{FDH}	mg cm^{-3}	0.01	0.01	3
t	min	0.5	0.5	200.0

F i g . 5 − *Optimization results. Average values of (a) DHPP, (b) NAD⁺, (c) formate,*
(d) LDH, and (e) FDH concentration, (f) time, (g) volumetric productivity
through generations and their standard deviation

sented in Figure 5. The values of optimization parameters are given as averages with standard deviations. Figure 5a, b, c, d, e, and f present the change in parameters through generations, and Figure 5 g presents the change in the average value of the target function (volumetric productivity). The initial generation was given randomly by genetic algorithm. Simulation results include 5 % error. Optimization was carried out through 60 generations of 8 individuals. Obtained optimal conditions are shown in Table 3.

T a b l e 3 − *Optimal initial conditions for the batch reactor experiment*

Optimal initial conditions		
c_{DHPP}	[mmol dm^{-3}]	4.69
c_F	[mmol dm^{-3}]	36.85
c_{NAD^+}	[mmol dm^{-3}]	4.95
γ_{LDH}	[mg cm^{-3}]	3
γ_{FDH}	[mg cm^{-3}]	2.94
t	[min]	8.5
Q_p	g dm^{-3} d^{-1}	93.06

Simulation results

For better understanding of the system's behaviour several simulations of the reaction system in the batch reaction conditions were carried out using the mathematical model. The simulations are carried out at the optimal conditions (Figure 6). Figure 6a presents the dependence of the volumetric productivity on the concentration of, both, enzymes (LDH and FDH) in the defined parameter range. In the concentration area of LDH from 0–3 mg cm^{-3}, it was found that this enzyme was the limiting factor in the optimization, which can be seen from its optimal concentration at upper border of the investigated area. Namely, LDH is very expensive and therefore it is not reasonable to consider greater
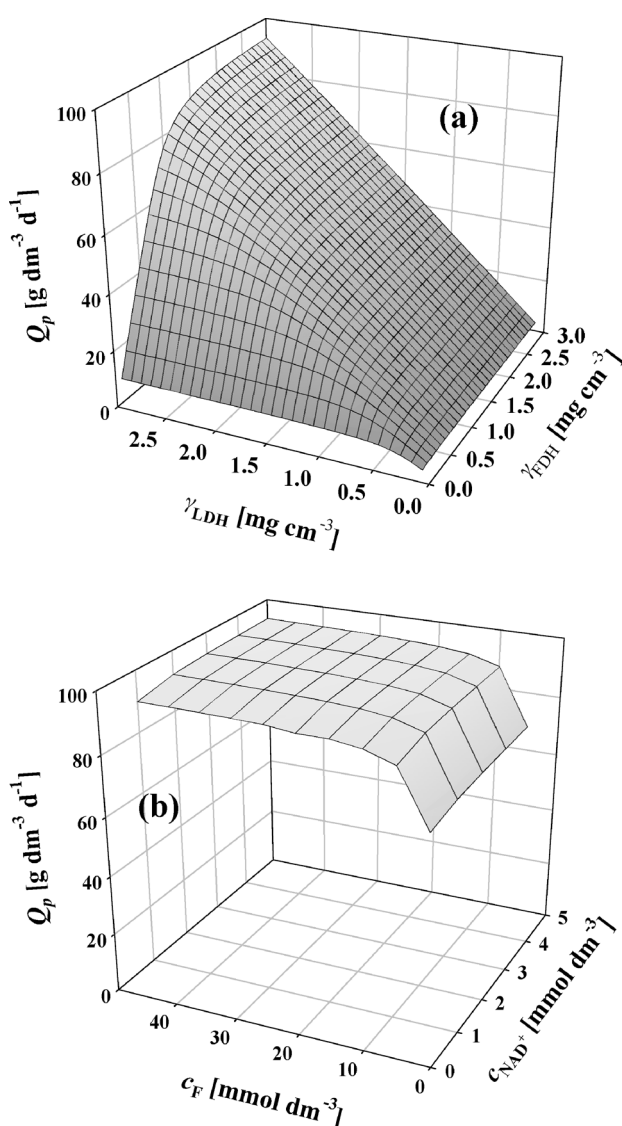
concentrations. As for FDH, which is concerned, its concentration has a significant influence on the target function up to 1.5 mg cm^{-3}, and beyond this value its influence can be neglected. The effect of formate and NAD$^+$ concentration on the volumetric productivity is considered in Figure 6b. Concentrations of formate higher than 20 mmol dm^{-3} do not have significant effect on the increase of the volumetric productivity. The same is with the concentrations of NAD$^+$ higher than 2 mmo dm^{-3}.

Conclusions

A mathematical model for the DHPL synthesis coupled by NADH regeneration system in the batch reactor was developed and verified by the associated experiment. Initial conditions of the reactions system were optimized in order to achieve maximal volumetric productivity. Genetic algorithm was used in this purpose. It was found to be an efficient tool for this system with 6 interdependent parameters, because it searches the parameter space independently. Optimal initial conditions were found to be: c_{DHPP} = 4.69 mmol dm^{-3}, c_F = 36.85 mmol dm^{-3}, c_{NAD^+} = 4.95 mmol dm^{-3}, γ_{LDH} = 3 mg cm^{-3}, γ_{FDH} = 2.94 mg cm^{-3}, t = 8.5 min. At these conditions volumetric productivity of 93.06 g dm^{-3} d^{-1} could be achieved.

This paper is an example of how mathematical modelling of enzyme kinetics and stochastic optimization method can be used in the search of the optimal reaction conditions.

Fig. 6 – *Dependence of volumetric productivity on the concentration of (a), both, enzymes LDH and FDH (c_{DHPP} = 4.69 mmol dm^{-3}, c_F = 36.85 mmol dm^{-3}, c_{NAD^+} = 4.95 mmol dm^{-3}, t = 8.5 min, (b) NAD$^+$ and formate (c_{DHPP} = 4.69 mmol dm^{-3}, γ_{LDH} = 3.0 mg cm^{-3}, γ_{FDH} = 2.94 mg cm^{-3}, t = 8.5 min)*

List of symbols

r_1 – reaction rate of the main reaction of DHPP reduction (μmol min^{-1} mg^{-1})

r_2 – reaction rate of the regeneration reaction (μmol min^{-1} mg^{-1})

V_m – maximal reaction rate (μmol min^{-1} mg^{-1})

c – concentration of substrate (mol dm^{-3})

M – molecular weight (g mol^{-1})

γ – mass concentration of enzyme (mg cm^{-3})

List of abbreviations

DHPP – 3,4-dihydroxyphenylpyruvate

DHPL – 3,4-dihydroxyphenyllactic acid

D-LDH and LDH – D-lactate dehydrogenase

FDH – formate dehydrogenase

GA – genetic algorithm

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