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Comparison of the L-malic acid production by isolated fumarase and fumarase in permeabilized baker's yeast cells

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Abstract

Production of L-malic acid from fumaric acid was performed by the action of the fumarase isolated from porcine heart and the fumarase in permeabilized baker's yeast cells. For each biocatalyst kinetic parameters were determined by measuring the initial reaction rate, and confirmed in the batch experiments. It was found that this reaction is inhibited by the product. Equilibrium conversion of about 80% was achieved in a batch mode by both biocatalysts. No by-product was detected during the L-malic production by permeabilized yeast cell. It was calculated by using the proposed model and experimentally verifies that 10 mg isolated fumarase gives the identical productivity in this biotransformation as 94.4 g of baker's yeast.

Keywords: L-Malic acid; Fumarase; Baker's yeast; Permeabilization; Modelling

1. Introduction

Biotransformations, using either the whole cells or isolated enzyme as biocatalysts, have been extensively applied [1,2]. They are mainly used in the pharmaceutical and agrochemical industries, because of the great need for optical pure molecules [3,4]. With the use of biocatalysts, tedious blocking and deblocking steps that are common in enantioselective and regioselective organic chemistry can be omitted [5].

The use of whole cell biocatalysts over purified enzymes is an advantage in terms of cost, isolation, and stability [6]. Also during the product isolation it is generally easier to separate whole cells than the isolated enzymes from reaction mixture. Whole cells removing include simple techniques like microfiltration or centrifugation, while to remove enzymes more sophisticate and energy demanding method like ultrafiltration must be used. Despite of these advantages the use of whole cells is limited because of substrate and product diffusion through the membrane, and unwanted side reactions due the presence of other enzymes in cells [7]. These disadvantages could be avoided by the permeabilization treatment. Permeabilization of the cells enables fast diffusion of the substrate/product across the cell membrane. It also removes most of the small molecular weight cofactors from the cell thus minimizing the unwanted side reactions catalyzed by enzymes that need cofactors [8].

L-Malic acid is an intermediate metabolite in the tricarboxylic acid (TCA) cycle of living cells [9]. Because it causes greater tartness and taste retention than other food acids, it is commonly used as a food additive [10]. Besides food industry, malic acid has also its application in the pharmaceutical and cosmetic industry [11]. In medicine only L-enantiomer of this acid is efficient in the treatment of liver dysfunction and hyperammonemia [12]. L-Malic acid can be prepared by isolating it from natural fruit juices or by separating it from the racemic mixture produced by chemical synthesis. Industrially, it is formed enzymatically from fumaric acid by the action of the enzyme fumarase. For that purpose microbial cells that contain fumarase are employed. In 1974 an industrial process in which whole cells of *Brevibacterium ammoniagenses* with high fumarase activity were immobilized in gel matrices of polyacrylamide was developed by Yamamoto et al. [13]. In 1977 productivity of this process, as well as operation stability were improved by changing *B. ammoniagenses* cells with the *B. flavum* [14,15]. Also immobilization process with polyacrylamide was changed to the more economical κ–carrageenan immobilization method [16]. A disadvantage of this system was that by-product like succinic acid

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Nomenclature

A_S	specific activity (U mg^{-1}, U $g_{ww}$$^{-1}$)
c	molar concentration (mmol dm^{-3})
K_i	inhibition constant (mmol dm^{-3})
K_m	Michaelis–Menten constant (mmol dm^{-3})
q	flow rate (cm^3 min^{-1})
q_v	volume productivity (g dm^{-3} day^{-1})
r	reaction rate (U cm^{-3}, U mg^{-1}, U $g_{ww}$$^{-1}$)
t	time (min)
T	temperature (°C)
V	volume (cm^3)
V_m	maximal reaction rate (U mg^{-1}, U $g_{ww}$$^{-1}$)
X	conversion (%)

Greek symbols

γ	mass concentration (g_{ww} dm^{-3}, mg dm^{-3})
λ	wavelength (nm)

was formed in considerable amounts. Detergents such as bile extract, bile acid and deoxychloic acid were found to reduce the amount of succinic acid formed by the whole cells [17].

In 1990s production of L-malic acid from fumaric acid using *Saccharomyces cerevisiae* cells was extensively studied. Figueiredo and Carvalho [18] have shown that entrapped *Saccharomyces cerevisiae* into polyacrylamide gel discs can produce malic acid form fumaric acid without formation of by-product. Peleg et al. [19] amplified the yeast *S. cerevisiae* for fumarase by cloning the fumarase gene of the same strain into an expression vector. Afterward Neufeld et al. [10] studied the kinetics of the bioconversion with this free and immobilized transformed strain and achieved a very high conversion of fumaric to L-malic acid without succinic acid formation.

The effect of substrate concentration on the L-malic acid production and the time course of the bioconversion process by employing laboratory *S. cerevisiae* strain SHY2 were examined by Wang et al. [11]. The amount of accumulated L-malic acid increased proportionally with the increase of initial fumaric acid concentration suggesting that this biotransformation is not substrate inhibited. Rosenberg et al. [12] have found out that the fumarase activity in the yeast strains of the genus *Dipodascus* was 10 times higher than that of *Saccharomyces cerevisiae* cells. Also the fumarase activity in yeast *Dipodascus* increased 100% with the Triton X-305 addition to the reaction mixture.

The latest work of the Bressler et al. [20] includes the biotransformation of fumaric to L-malic acid by immobilized yeast engineered to overproduce fumarase. In order to achieve a higher conversion this reaction was performed in a specific bioreactor that was divided into three compartments (feed, reaction and product) by two supported liquid membranes. The first membrane (between feed and reaction) was selective toward the substrate (fumaric acid), while the other one (between product and reaction) was selective toward the L-malic acid. Recently, in our lab we tested several commercial yeast strains. In order to achieve higher activity of the yeast cells we optimized the cell-pretreatment procedure by testing the different types and concentration of surfactants as well as the time of treatment. No side products were detected because by permeabilization treatment all coenzymes that were necessary for their production were removed from the cells [21].

The aim of this work was to develop the mathematical model of the biotransformation for the L-malic acid production from fumaric acid catalyzed by isolated enzyme and whole permeabilized cell as the biocatalysts. The modeling of the biotransformation as a tool for enzyme reaction engineering represents an important role in developing the enzyme-catalyzed reaction for large-scale production. It includes kinetic parameters estimation that can be used to find optimal operation points and to increase knowledge about the process and facilitate identification of the most effective reactor operation mode [22]. The developed mathematical model was used to calculate concentrations of the both biocatalysts that will result in the same productivity of the L-malic acid in batch reactor.

2. Experimental

2.1. Materials and microorganism

Fumaric acid, L-malic acid, hexadecyltrimethylammonium bromide (CTAB) and KH_2PO_4 were purchased from Fluka Chemie. Na_2HPO_4 and K_2HPO_4 were purchased from Kemika (Croatia). Fumarase from porcine heart (E.C. 4.2.1.2) was from Sigma. Fresh baker's yeast was purchased from Kvasac d.o.o (Croatia).

2.2. Biotransformations with isolated fumarase

2.2.1. Fumarase kinetics

The fumarase kinetics was measured by initial rate method at spectrophotometer (Shimadzu, UV 1601) at 290 nm in thermostated (30 °C) quartz cuvettes in a total volume of 1 cm^3 by monitoring the fumaric acid concentration. The activities were calculated using the molar extinction coefficient [23] of 0.11 cm^2 μmol^{-1}.

The optimum pH for the L-malic acid production was determined using the phosphate buffer in the pH range 5.4–8.0.

2.2.2. Bioreactor experiments with isolated fumarase

Enzymatic production of L-malic acid using isolated enzyme was performed in a glass batch reactor at different initial fumaric acid concentration ($c_{\text{fumaric acid},0}$ = 25; 50; 100; 200 mmol dm^{-3}). The operating volume was 10 cm^3 and the reactor was thermostated at 30 °C. The reaction was initiated by adding the fumarase solution (γ_{fumarase} = 8.01 mg dm^{-3}).

2.3. Whole cells biotransformations

2.3.1. Permeabilisation procedure

One gram (wet weight, ww) of yeast cells was suspended in 10 cm^3 0.2% (w/v) CTAB for 5 min with intermittent shaking [21,24]. The cells were then separated by centrifugation at 5000 rpm for 10 min. The yeast pellet was washed with phosphate buffer and suspended uniformly in 0.1 mol dm^{-3} potassium phosphate buffer pH 7.0.

2.3.2. Permeabilized yeast kinetics

The fumarase kinetics in permeabilized yeast cells was measured using initial rate method. Using the fumarate as a substrate, all the measurements were carried out in a 20 cm^3 reactor. The reactor was thermostated at 30 °C, and the permeabilized cell concentration was 5 g_{ww} dm^{-3}. Sampling was carried out every 30 s in a total time of 3 min and the reaction was stopped by addition of 0.1 mmol dm^{-3} NaOH. The fumaric acid concentration in the samples was determined by HPLC. For the product inhibition the fumaric acid concentration

of 200 mmol dm^{-3} was kept constant in each measurement, while L-malic acid concentration varied between 0 and 200 mmol dm^{-3}.

For the L-malic acid as a substrate measurements were carried out in thermostated (30 °C) quartz cuvettes in a total volume of 1 cm^3. The increase of fumarate was monitored at 240 nm by spectrophotometer. The permeabilized cells concentration in the cuvette was 1 g_{ww} dm^{-3} and initial rate was calculated by using a molar extinction coefficient [23] of 2.1715 cm^2 μmol^{-1}.

2.3.3. Whole cells bioreactor experiments

The experiments in the 500 cm^3 shake flasks were carried out at different concentrations of baker's yeast in order to validate the model. 100 cm^3 reaction solution was stirred on the shaker at 200 rpm and thermostated at 30 °C. Four sets of experiments were carried out at initial fumarate concentration of 50 mmol dm^{-3} and permeabilized cell concentration of 5, 10, 20 and 100 g_{ww} dm^{-3}, respectively. In order to make a comparison between pemeabilized cells and non-permeabilized one, a set of experiments was carried out with the initial fumarate concentration of 50 mmol dm^{-3} and 10 g_{ww} dm^{-3} of non-permeabilized yeast. Each set of experiment was repeated twice and mean value is presented. Using the permeabilized yeast cell the production of L-malic acid was also carried out in the fed-batch mode in a 2 dm^3 bioreactor. The reaction volume was 1 dm^3, the solution was stirred at 200 rpm and thermostated at 30 °C. The initial fumaric acid concentration was 50 mmol dm^{-3} and the concentration of permeabilized yeast cells was 10 g_{ww} dm^{-3}. In the fed-batch operating mode the 225 mmol dm^{-3} fumaric acid feed was set at 42 cm^3 h^{-1}. In the all experiments, fumaric acid was diluted in a 0.1 mol dm^{-3} phosphate buffer and pH was adjusted to 7 by NaOH.

2.4. HPLC analysis

The fumaric and and L-malic acid concentrations were analyzed with HPLC (Sykam, Shimadzu) using the reverse phase C_{18} column (Merck, LiChrosorb® RP-18, 5 μm, 125 mm × 4 mm) and the UV detector at 210 nm. The mobile phase was water of pH 2.10–2.15, adjusted with perchloric acid [25], at a flow rate of 0.7 ml min^{-1}. The analysis was performed at 30 °C.

2.5. The mathematical model and data handling

The kinetics of purified fumarase from porcine heart and fumarase in permeabilized baker's yeast cell for fumaric acid hydration, as for the reverse reaction of L-malic acid dehydration was described by Michaelis–Menten equation with competitive product inhibition (Table 1, Eqs. (1) and (2)). The mass balance equations for the L-malic acid production in batch (Eqs. (3) and (4)) and fed-batch reactor (Eqs. (5)–(7)) are given in the Table 1.

The kinetic parameters were estimated by non-linear regression analysis using the simplex or least squares method implemented in "Scientist" software package [26]. The numerical values of the parameters were evaluated by fitting the kinetic model (Table 1, Eqs. (1) and (2)) to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine and fed again to the integration step until minimal error between experimental and integrated values was achieved (built-in Scientist). The set of optimum parameters was used for the simulation according to the model equations (Table 1, Eqs. (3)–(7)). The residual sum of squares was defined as the sum of the squares of the differences between experimental and calculated data. The "Episode" algorithm for stiff system of differential equations, implemented in the "Scientist" software package, was used for the simulations. It uses variable coefficient Adams-Moulton and Backward Differentiation Formula methods in the Nordsieck form, treating the Jacobian matrix as full or banded.

3. Results and discussion

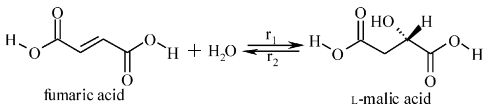
3.1. Fumarase kinetics

Prior to measuring the kinetic of enzyme fumarase from porcine heart, the optimum pH for the L-malic acid production was determined. Using the 15 mmol dm^{-3} solution of fumaric and malic acid in phosphate buffer the fumarase activity was measured as a pH function. The results (Fig. 1) show that the maximum enzyme activity for fumarate hydration was achieved at pH 7, while for L-malic dehydration it was at pH 8. As the latter reaction is unwanted, pH 7 was used in the further research.

As the biotransformation of fumaric to L-malic acid is reversible, the kinetic for the L-malic dehydration reaction was also measured. The initial reaction rate dependence on the fumaric acid, as well as on the L-malic acid concentration was determined. The impact of L-malic acid on the initial reaction rate of the fumaric acid hydration as the fumaric acid to the L-malic acid dehydration was also measured.

It was found that the malic acid inhibits hydration of fumaric acid for both types of biocatalyst, while product inhibition in the reverse reaction was found only in a case of isolated enzyme (Figs. 2 and 3). According to the work of Marconi et al. [27], these phenomena were described as formal competitive inhibition with Eqs. (1) and (2) (Table 1).

Table 1
Kinetic and mass balance equations of the batch and fed-batch reactor for the L-malic production



Kinetic equations

$$r_1 = \frac{V_m^{\text{fumaric acid}} \gamma_{\text{biocatalyst}} c_{\text{fumaric acid}}}{K_m^{\text{fumaric acid}}(1 + c_{\text{malic acid}}/K_i^{\text{malic acid}}) + c_{\text{fumaric acid}}} \quad (1)$$

$$r_2 = \frac{V_m^{\text{malic acid}} \gamma_{\text{biocatalyst}} c_{\text{malic acid}}}{K_m^{\text{malic acid}}(1 + c_{\text{fumaric acid}}/K_i^{\text{fumaric acid}}) + c_{\text{malic acid}}} \quad (2)$$

Mass balance equations – batch reactor

$$\frac{dc_{\text{fumaric acid}}}{dt} = r_2 - r_1 \quad (3)$$

$$\frac{dc_{\text{malic acid}}}{dt} = r_1 - r_2 \quad (4)$$

Mass balance equations – fed-batch reactor

$$\frac{dc_{\text{fumaric acid}}}{dt} = \frac{-c_{\text{fumaric acid}} + c_{0,\text{fumaric acid}}}{V} q_{c_0} + r_2 - r_1 \quad (5)$$

$$\frac{dc_{\text{malic acid}}}{dt} = \frac{-c_{\text{malic acid}}}{V} q_{c_0} + r_1 - r_2 \quad (6)$$

$$\frac{dV}{dt} = q_{c_0} \quad (7)$$

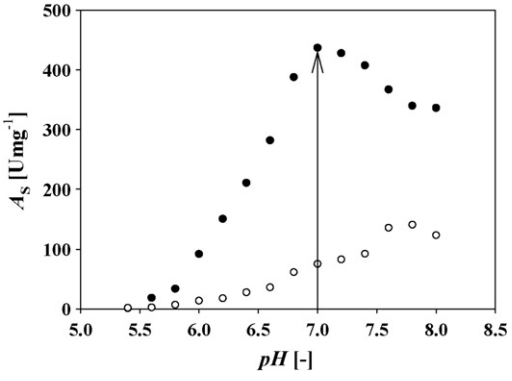


Fig. 1. Effect of the pH on the isolated fumarase activity with L-malic and fumaric acid as a substrate ($c_{\text{fumaric acid}}$ = 15 mmol dm^{-3}, $c_{\text{malic acid}}$ = 15 mmol dm^{-3}; γ_{fumarase} = 6.24 mg dm^{-3}; 0.1 mmol dm^{-3} phosphate buffer; T = 30 °C; λ = 290 nm), ● fumaric acid, ○ L-malic acid.

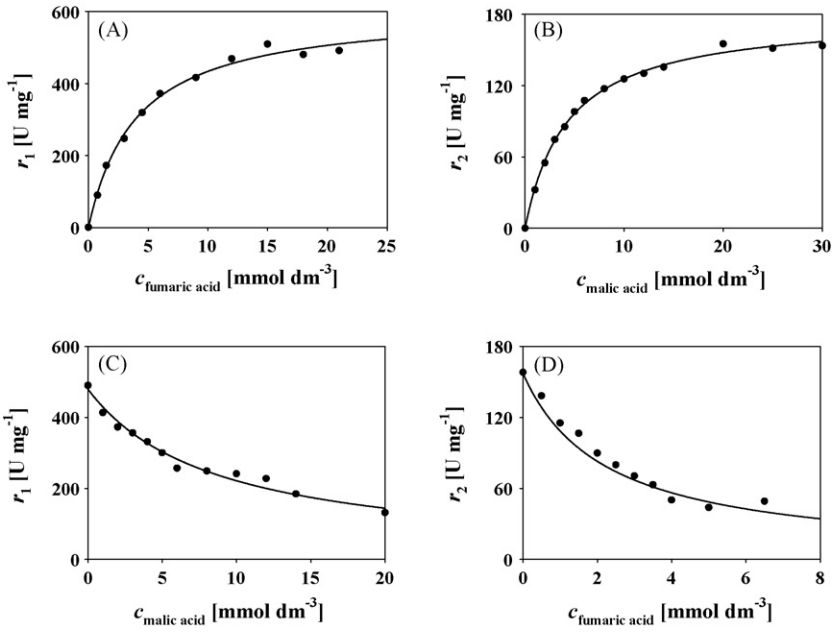


Fig. 2. Kinetics of the isolated fumarase ($\gamma_{\text{fumarase}} = 6.24\,\text{mg dm}^{-3}$; 0.1 mmol dm^{-3} phosphate buffer; $T = 30\,^{\circ}\text{C}$; $\lambda = 290\,\text{nm}$): (A) varying of fumaric acid concentration; (B) varying of L-malic acid concentration; (C) varying of L-malic acid concentration as inhibitor at constant concentration of fumaric acid ($c_{\text{fumaric acid}} = 15\,\text{mmol dm}^{-3}$); (D) varying of fumaric acid concentration as inhibitor at constant concentration of L-malic acid ($c_{\text{malic acid}} = 25\,\text{mmol dm}^{-3}$); (●) experiment; (—) model.

Using the experimental data, the kinetic parameters presented in Table 2 were estimated by non-linear regression analysis.

The purified enzyme shows about three times higher maximal activity to fumaric acid as a substrate then to L-malic acid at pH 7, while according to Marconi et al. [27] at pH 7.3 the ratio of maximal activities is about 2.5. The data for Michaelis–Menten constants of fumarase isolated from pig heart presented here are consistent with the studies of Cernia et al. [28]. Inhibition of L-malic acid in the reaction of fumaric acid hydration is about six times lower than the product inhibition in the reverse reaction (Table 2). Obtained kinetic data indicate that this reaction is shifted in the direction of the L-malic acid production.

Maximal activities of enzyme in permeabilized yeast cells indicate that the reaction of the fumaric acid hydration is around four times faster than the reverse reaction. Also the

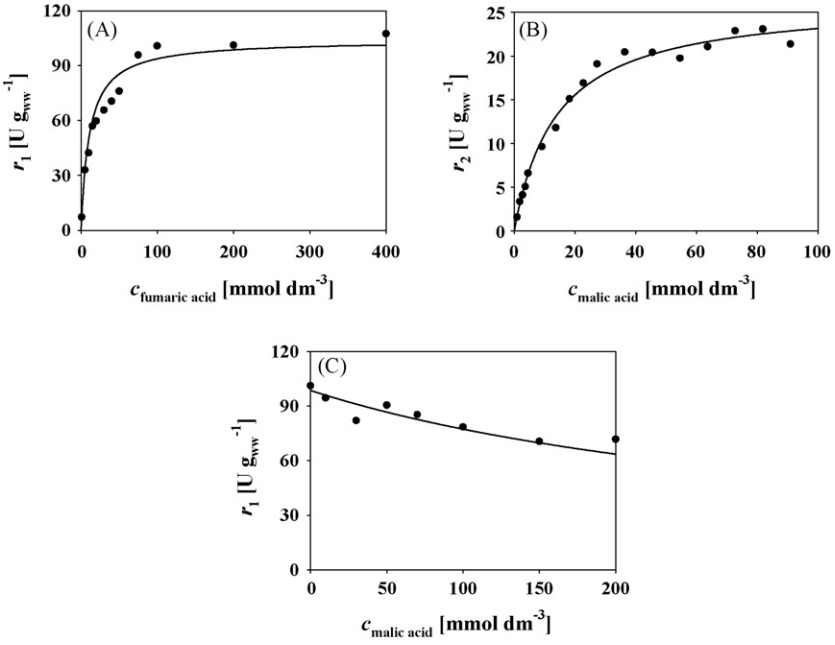


Fig. 3. Kinetics of the permeabilized yeast cells (0.1 mmol dm^{-3} phosphate buffer; $T = 30\,^{\circ}\text{C}$): (A) varying of fumaric acid concentration ($\gamma_{\text{yeast}} = 5\,g_{ww}\,\text{dm}^{-3}$; $V_{\text{reactor}} = 20\,\text{cm}^3$); (B) varying L-malic acid concentration ($\gamma_{\text{yeast}} = 1\,g_{ww}\,\text{dm}^{-3}$; $\lambda = 240\,\text{nm}$); (C) varying of L-malic acid concentration as inhibitor at constant concentration of fumaric acid ($\gamma_{\text{yeast}} = 5\,g_{ww}\,\text{dm}^{-3}$; $V_{\text{reactor}} = 20\,\text{cm}^3$; $c_{\text{fumaric acid}} = 200\,\text{mmol dm}^{-3}$); (●) experiment; (—) model.

Table 2
Kinetic parameters of isolated fumarase and fumarase in permeabilized yeast cells

Parameters	Estimated values	
	Fumarase from porcine heart	Fumarase in permeabilized yeast cells
Fumaric acid hydration		
$V_m^{\text{fumaric acid}}$	607.214 ± 17.220 U mg^{-1}	104.029 ± 5.321 U $g_{ww}$$^{-1}$
$K_m^{\text{fumaric acid}}$	3.999 ± 0.364 mmol dm^{-3}	10.902 ± 0.897 mmol dm^{-3}
$K_i^{\text{malic acid}}$	0.279 ± 0.031 mmol dm^{-3}	18.775 ± 1.556 mmol dm^{-3}
L-Malic acid dehydration		
$V_m^{\text{malic acid}}$	179.456 ± 2.607 U mg^{-1}	26.379 ± 0.724 U $g_{ww}$$^{-1}$
$K_m^{\text{malic acid}}$	4.286 ± 0.199 mmol dm^{-3}	13.912 ± 1.297 mmol dm^{-3}
$K_i^{\text{fumaric acid}}$	1.807 ± 0.093 mmol dm^{-3}	–

L-malic acid inhibition in that reaction is not high, which points out that the equilibrium with this biocatalyst would also be in the direction of the L-malic production (Table 2). Activity for fumaric acid hydratation assigned in this work (20.8 mmol h^{-1} g^{-1}) is about 15 times higher than the one obtained with the cells of *Saccharomyces cerevisiae* treated with malonate and sodium dodecyl sulfate (1.218 mmol h^{-1} g^{-1}), but three times lower for the same cells immobillized within polyacrylamide gel beads (60.6 mmol h^{-1} g^{-1}) [29]. Activity of the cells presented here are in good accordance with the yeast *Dipodascus magnusii* treated with fumarate and Triton X-305 (18.78 mmol h^{-1} g^{-1}) [12] and around three times higher than that described by Yamamoto et al. for immobilized *B. ammoniagenes* cells (7.48 mmol h^{-1} g^{-1} of cell pretreated with bile extract) [13].

Furthermore, K_m parameter of the fumaric acid is higher for the cells permeabilized with malonate and sodium dodecyl sulfate (38.3 mmol dm^{-3}) [29] which could be addressed to the better cell permeability achieved by permeabilization with CTAB. To complete mathematical model for the L-malic acid production by these two biocatalysts, balance equations for the production in the batch and fed-batch mode were proposed (Table 1).

3.2. L-Malic acid production by purified enzyme fumarase—model validation

To validate the proposed model for the batch production of L-malic acid (Table 1, Eqs. (1)–(4)) by isolated fumarase from porcine heart, several batch experiments with different initial fumaric acid concentration were carried out. The comparison of experimental results and simulation results using estimated parameters (Table 2) shows that proposed mathematical model describes well the production of L-malic acid (Fig. 4A). It could be seen that the equilibrium of the reaction is shifted in the direction of the L-malic production, and conversion of 80% was achieved.

3.3. Batch process for the L-malic acid production using permeabilized yeast cells—model validation

Proposed mathematical model for the batch production process of L-malic acid by permeabilized yeast cells was verified by several experiments that were carried out at the different concentration of baker's yeast (Fig. 4B). The same conversion of around 80% was achieved as with the isolated enzyme. It is also important to mention that in these experiments no by-product such as a succinic acid and oxalic acid was detected and the sum of fumaric and L-malic concentration was constant. Obviously cofactors essential to the formation of succinic and oxalic acid are released from the cells during the permeabilization of cell wall [18].

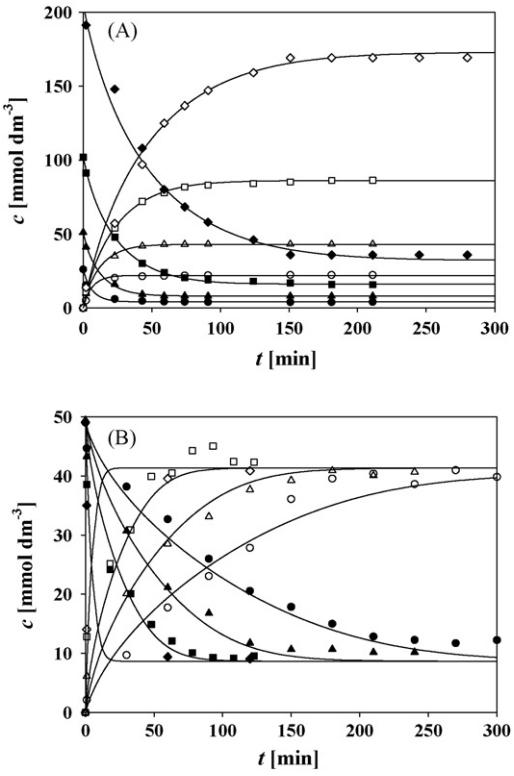


Fig. 4. Production of L-malic acid in the batch mode by: (A) isolated fumarase (γ_{fumarase} = 8.01 mg dm^{-3}; 0.1 mmol dm^{-3} phosphate buffer; T = 30 °C; V_{reactor} = 10 cm^3; $c_{\text{fumaric acid},0} \approx$ ●○ 25 mmol dm^{-3}, ▲△ 50 mmol dm^{-3}, ■□ 100 mmol dm^{-3}, ◊♦ 200 mmol dm^{-3}); (B) permeabilized yeast cells ($c_{\text{fumaric acid},0} \approx$ 50 mmol dm^{-3}; 0.1 mmol dm^{-3} phosphate buffer; T = 30 °C; V_{reactor} = 100 cm^3; γ_{yeast} = ●○ 5 g_{ww} dm^{-3}, ▲△ 10 g_{ww} dm^{-3}, ■□ 20 g_{ww} dm^{-3}, ◊♦ 100 g_{ww} dm^{-3}); black symbols – fumaric acid; white symbols – L-malic acid; (—) model.

3.4. Comparison of L-malic production by permeabilized and non-permeabilized yeast cells

Experiment with non-permeabilized baker's yeast cells was carried out to define the influence of the permeabilization treatment to the bioconversion process. Comparison of experiments by permeabilized and non-permeabilized cells that were conducted in the batch mode using the same initial conditions (fumaric acid and yeast cells concentration) is shown in a Fig. 5.

Very low conversion ($X = 10.9\%$) after 2.5 h yielded low volume productivity ($q_v = 7.1\ g\ dm^{-3}\ day^{-1}$) was assigned in the L-malic production with non-permeabilized cells compared to those achieved by permeabilized yeast cells at the same time ($X = 79.4\%, q_v = 50.5\ g\ dm^{-3}\ day^{-1}$). The reason for that is a significantly small reaction rate with these cells which is controlled by the product and substrate mass transfer. This experiment has shown that bioconversion with non-permeabilized cells is possible, but the obtained productivity is negligible.

3.5. Comparison of L-malic acid production by isolated fumarase and permeabilized yeast cells

In batch production of L-malic acid with $10\ mg\ dm^{-3}$ isolated fumarase the conversion of around 80% and productivity of $13.5\ g\ dm^{-3}\ min^{-1}$ was achieved (Fig. 6).

Proposed mathematical model (Table 1, Eqs. (1)–(4)) with the estimated parameters (Table 2) for the permeabilized cells was used to calculate the baker's yeast concentration that would result the same conversion and productivity in the same time as with $10\ mg\ dm^{-3}$ isolated fumarase. The experiment was performed by calculated amount of permeabilized yeast cells, which was $\gamma_{yeast} = 94.4\ g_{ww}\ dm^{-3}$, at the identical conditions as with the isolated enzyme. The results of these two experiments and the model simulations have shown that the proposed model could predict with high accuracy the productivity during the L-malic acid production (Fig. 6). It is also demonstrated that with 94.4 g baker's yeast, it is possible to obtain the same productivity as with 10 mg of isolated fumarase in a fumaric acid biotransformation.

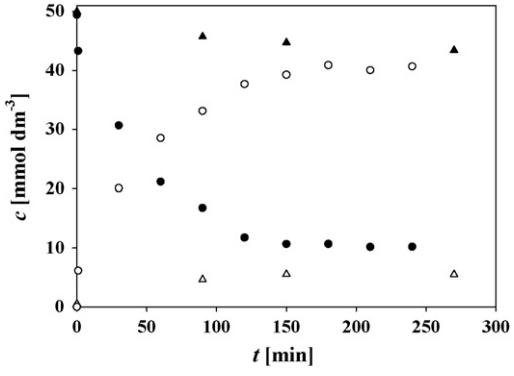


Fig. 5. Comparison of L-malic acid production in the batch mode by permeabilized and non-permeabilized yeast cells ($c_{fumaric\ acid,0} \approx 50\ mmol\ dm^{-3}$; $0.1\ mmol\ dm^{-3}$ phosphate buffer; $T = 30\ °C$; $V_{reactor} = 100\ cm^3$; $\gamma_{yeast} = 10\ g_{ww}\ dm^{-3}$); (●○) permeabilized cells; (▲△) non-permeabilized cells; black symbols – fumaric acid, white symbols – L-malic acid.

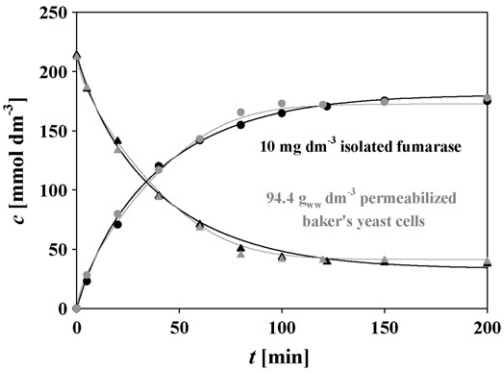


Fig. 6. Comparison of L-malic acid production in the batch mode ($0.1\ mmol\ dm^{-3}$ phosphate buffer; $T = 30\ °C$; $V_{reactor} = 100\ cm^3$) by isolated fumarase ($\gamma_{fumarase} = 10\ mg\ dm^{-3}$) and permeabilized yeast cells ($\gamma_{yeast} = 94.4\ g_{ww}\ dm^{-3}$), circles – L-malic acid, triangles – fumaric acid, black symbols – isolated fumarase, grey symbols – permeabilized yeast cells.

As the source of whole cell biocatalyst in this work was baker's yeast (bought in the local store), this example shows how yeast biomass that are usually a waste in industries like wine and brewing can be utilized for performing a biotransformation.

3.6. Fed-batch process for the L-malic acid production using permeabilized yeast cells

Results of the fed-batch mode experiment by permeabilized yeast cells are illustrated in the Fig. 7. Experiment was carried out in a fed-batch mode after 185 min when the fumaric acid feed started ($c_{fumaric\ acid} = 225\ mmol\ dm^{-3}$, $q = 0.7\ cm^3\ min^{-1}$; phase II). Until that time L-malic acid was produced in a batch mode (phase I). The dependence of the conversion on time (Fig. 7B) shows that the conversion during the L-malic production in the fed-batch mode has decreased to 70%, which means that at higher L-malic acid concentration the reverse reaction rate (Fig. 3B), as well as L-malic inhibition in forward reaction (Fig. 3C) has impact on the fumaric acid conversion in this type of reactor. This fact is also well described by the proposed model.

From the industrial point of view an equilibrium conversion such as was presented in this work is a general problem for the L-malic acid production. Namely, enzymatic production of L-malic acid is an equilibrium process and the maximal achieved conversion of fumaric acid is usually between 80% and 90% (e.g. *S. cerevisiae* amplified for fumarase have a conversion of 88% (pH 7.5, 37 °C) [19], *B. flavum* 83.3% (pH 7, 37 °C) [15], *B. ammoniagenes* 82.8% (pH 7, 37 °C) [13]). The product mixture of malic and fumaric acid is hard to separate due to similar chemical and physical properties of both compounds. Several ways were proposed to overcome this problem. Integrated systems to recover unreacted fumaric acid in situ by exploiting its small solubility at low pH are usual proposed solution for reach higher conversions in this system [30–34]. Recently liquid membranes consisting of organophosphorus extractant selective toward fumaric and L-malic acid were used for continuous biotransformation of fumaric to L-malic acid and a conversion of

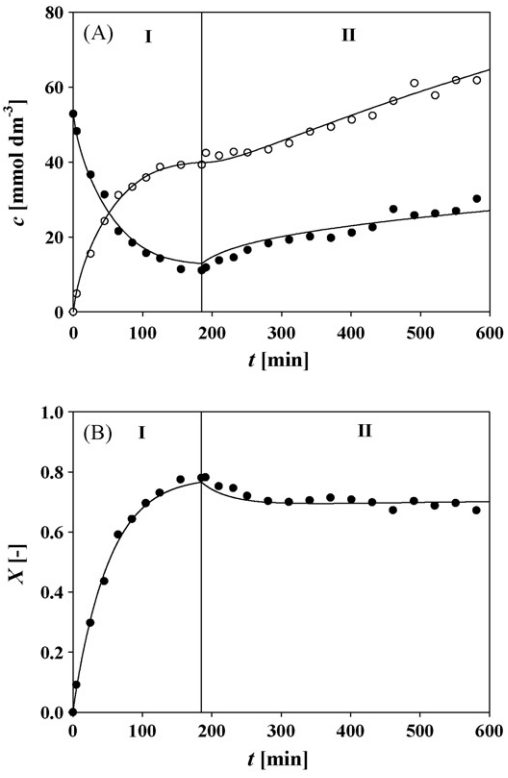


Fig. 7. Production of L-malic acid in the fed-batch mode by permeabilized yeast cells ($\gamma_{yeast} = 10\ g_{ww}\ dm^{-3}$; $c_{fumaric\ acid,0} = 225\ mmol\ dm^{-3}$; $q = 0.7\ cm^3\ min^{-1}$; 0.1 mmol dm^{-3} phosphate buffer; $T = 30\ ^\circ C$; $V_{reactor} = 1000\ cm^3$); (A) fumaric and L-malic acid concentration time changes ((●) fumaric acid; (○) L-malic acid; (—) model); (B) fumaric acid conversion time change ((●) experiment; (—) model).

100% was achieved [20]. Since the main disadvantage of this system is membrane instability, a hint for the future work could be improving its stability before integrating it in the system for the L-malic production.

4. Conclusion

Isolated fumarase and permeabilized baker's yeast cells were used to carry out the biotransformation of fumaric to L-malic acid. The proposed mathematical model with estimated parameters describes well the L-malic acid production by both biocatalysts. Batch experiment carried out with non-permeabilized cells showed that permeabilization treatment significantly increases the rate of L-malic acid production by the yeast cells. By using a validated model it was possible to calculate the concentration of isolated fumarase and permeabilized baker's yeast cells that would achieve the same productivity in the investigated biotransformation.

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