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Coenzyme regeneration catalyzed by NADH oxidase from *Lactobacillus brevis* in the reaction of L-amino acid oxidation

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Abstract

In this paper L-methionine oxidation catalyzed by L-phenylalanine dehydrogenase from *Rhodococcus* sp. M4 was studied. It was found that the reaction equilibrium is shifted to the side of reduction, and it was therefore necessary to regenerate NAD^+ to increase L-methionine conversion. NADH oxidase from *Lactobacillus brevis* was used for that purpose. The enzyme was kinetically characterized. It was found that the enzyme is inhibited by NAD^+. Hence, NADH oxidation catalyzed by NADH oxidase was described by the Michaelis–Menten equation which included anticompetitive NAD^+ inhibition. L-Methionine oxidation was described by formal double-substrate Michaelis–Menten model which included competitive product inhibition by NADH. 2-Oxo-4-methylthiobutyric acid reduction was described by formal three-substrate Michaelis–Menten kinetics which included competitive inhibition by NAD^+. Experiments were carried out in the batch and in the continuously operated enzyme membrane reactor. 100% L-methionine conversion was achieved in the batch reactor. The conversion was lower in the continuously operated enzyme membrane reactor where enzyme deactivation occurred.

Keywords: Amino acid; Coenzyme regeneration; NADH oxidase; L-Phenylalanine dehydrogenase; Enzymes; Enzyme biocatalysis

1. Introduction

There are various methods available for the stereoselective synthesis of optically pure amino acid [1]. One of these methods includes the use of L-amino acid dehydrogenases. Using these enzymes the synthesis of L-amino acid can be carried out from an α-keto acid as a starting material, while D-amino acid can be synthesized from the corresponding racemate, by complete oxidation of L-amino acid followed by separation of an α-keto acid [2]. Besides enantiomerically pure amino acids as valuable products, L-amino acid dehydrogenases can be used for an α-keto acid synthesis. It is known that certain α-keto acids are valuable as pharmaceuticals [3,4] and nutraceuticals [5]. In all cases coenzyme regeneration [6] system is necessary to ensure the equilibrium shifts towards the wanted products. In the case of L-amino acid synthesis from the corresponding α-keto acid formate dehydrogenase [7,8] can be used for NADH regeneration, while in the case of L-amino acid oxidation an efficient method must be found for NAD^+ regeneration. NADH oxidase is the most promising enzyme for that purpose, even though it is not commercially available, unlike formate dehydrogenase. It has been used before in two systems that involve amino acid dehydrogenase [9,10]. The first example is the synthesis of D-*tert*-leucine from the corresponding racemate [9], and the second example, the synthesis of α-ketoglutarate from L-glutamate [10]. NADH oxidases from different origins (*Lactobacillus brevis* and *Lactobacillus sanfranciscensis*) were used. They were both quite successful in coenzyme regeneration and equilibrium shifting. Besides these examples, NADH oxidases can be used for NAD^+ regeneration in oxidations catalyzed by alcohol dehydrogenases [11–13]. There are various NADH oxidases known, some of which produce hydrogen peroxide as a by-product [14–16], while others produce water [10–13,17–19].

In our case NADH oxidase from *L. brevis* was used for coenzyme regeneration in the reaction of L-methionine oxidation catalyzed by L-phenylalanine dehydrogenase from *Rhodococcus* sp. M4 [20]. This reaction was found to be thermodynamically unfavored and could not be carried out without an efficient regenerating system. With NADH oxidase present in the reaction system, L-methionine could be completely oxidized and therefore employed for D-methionine synthesis from the corresponding racemate. Since L-phenylalanine dehydrogenase

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Nomenclature

c	concentration (mmol dm^{-3})
k_d	deactivation constant of the enzyme (min^{-1})
K_m	Michaelis–Menten constant (mmol dm^{-3})
K_i	product inhibition constant (mmol dm^{-3})
L-met	L-Methionine
L-PheDH	L-Phenylalanine dehydrogenase
r_1	reaction rate of L-PheDH catalyzed L-methionine oxidation (U cm^{-3})
r_2	reaction rate of L-PheDH catalyzed 2-oxo-4-methylthiobutyric acid reduction (U cm^{-3})
r_3	reaction rate in the reaction of NADH oxidase catalyzed NADH oxidation (U cm^{-3})
V	volume (ml)
V_m	maximal reaction rate (U mg^{-1})
2-oxo	2-oxo-4-methylthiobutyric acid

Greek letter

γ	concentration (mg cm^{-3})

accepts other amino acids as substrates, the same system can be used for the production of other optically pure D-amino acids, as it was in the case of D-*tert*-leucine mentioned earlier where L-leucine dehydrogenase was used [9]. It is known that D-amino acids are widely used in the pharmaceutical industry as precursors of various physiologically active compounds [21,22]. Synthesis of the corresponding α-keto acids in these systems can be carried out as well.

The mathematical model of the reaction studied in this paper (Fig. 1) was developed. Enzyme kinetics of both enzymes was studied in detail by using the initial reaction rate method, and the influences of all the reaction compounds on both enzymes were examined. The developed mathematical model consisting of enzyme reaction kinetics and mass balances in reactor was validated by the batch reactor experiments. This detailed knowledge of the reaction system is essential for the reactor development as well as for collecting the knowledge of the catalyst under operational conditions [23]. Theoretical modeling of enzyme kinetics and reactors is useful for finding the optimal operation points [24] as well as for the identification of the most effective reactor mode [25].

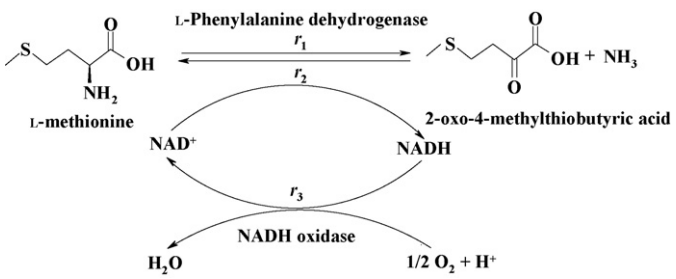

Fig. 1. Reaction scheme of L-methionine oxidation catalyzed by L-phenylalanine dehydrogenase with coenzyme regeneration by NADH oxidase from *Lactobacillus brevis*.

2. Experimental

2.1. Materials

L-Phenylalanine dehydrogenase was from *Rhodococcus* sp. M4, NAD^+ and NADH were from Jülich Fine Chemicals (Jülich, Germany). L-Methionine and trishydroxymethylaminomethane were from Fluka (Buchs, Switzerland), 2-oxo-4-methylthiobutyric acid was from Sigma (Schnelldorf, Germany). NADH oxidase from *L. brevis* was a gift from Prof. Hummel (IMET, Research Center Jülich, Germany) and was prepared as described elsewhere [11].

2.2. Enzyme activity assays

2.2.1. L-Phenylalanine dehydrogenase assay

L-Phenylalanine dehydrogenase (L-PheDH) activity was measured according to the standard enzymatic assay which is based on NADH concentration followed at 340 nm [26]. The reaction mixture contained substrate (L-methionine, 2-oxo-4-methylthiobutyric acid, ammonium formate) solution, NAD^+ or NADH solution in 0.2 mol dm^{-3} Tris–HCl buffer and L-PheDH solution. Activities were calculated from the absorbance change at 340 nm, using a molar extinction coefficient for NADH of 6.22 cm^2 μmol^{-1}. All measurements were carried out in quartz cuvettes of 1 cm^3 and at 30 °C. One Unit of L-phenylalanine dehydrogenase was defined as the amount of enzyme necessary to oxidize 1 μmol of L-methionine per minute at 30 °C and in 0.2 mol dm^{-3} Tris–HCl buffer, pH 9.0.

2.2.2. NADH oxidase assay

NADH oxidase activity was followed via spectrofotometer at 340 nm in quartz cuvettes of 1 cm^3 at 30 °C [27]. Measurements were carried out in Tris–HCl buffer pH 9.0 in a total volume of 1 cm^3. Solutions required for the assay were: NADH and NAD^+ solution in Tris–HCl buffer and NADH oxidase solution. L-Methionine and 2-oxo-4-methylthiobutyric acid solutions were used to evaluate their inhibiting effect on this enzyme. One Unit of NADH oxidase was defined as the amount of enzyme necessary to oxidize 1 μmol of NADH per minute at 30 °C and in 0.2 mol dm^{-3} Tris–HCl buffer, pH 9.0.

2.3. HPLC analysis

L-Methionine and 2-oxo-4-methylthiobutanoic acid were followed by HPLC (Sykam, Shimadzu) with a reverse phase C_{18} column (Lichrosorb 100, Merck, 125 mm × 4 mm, 5 μm) and UV detector at 210 nm. The mobile phase was water with the addition of perchloric acid until pH 2.10–2.15 was reached [28] at a flow rate of 0.9 cm^3 min^{-1}. The analysis was performed at 30 °C. Standard solutions were prepared by dissolving appropriate masses of the standard in hydrochloric acid (0.1 mol dm^{-3}). Samples taken from the reactor were diluted in hydrochloric acid (0.1 mol dm^{-3}) as well. Retention times of L-methionine and 2-oxo-4-methylthiobutanoic acid were 2.9 and 9.5 min, respectively.

2.4. Reactor experiments

L-Methionine oxidation was carried out in the batch reactor and continuously operated enzyme membrane reactor [24,25,29–32]. Two batch reactor experiments were carried out: without and with coenzyme regeneration. In all cases the reactor volume was 10 cm^3, and the temperature 30 °C. All experiments were carried out in 0.2 mol dm^{-3} Tris–HCl buffer pH 9.0. The continuous reactor set-up consisted of an alternating piston pump, injection septum, enzyme membrane reactor, magnetic stirrer and thermostat. An Amicon membrane (regenerated cellulose, cut off 10 kDa) placed in the reactor was used to retain enzymes in the enzyme membrane reactor during the experiment. The reaction was started by adding the enzymes through the injection septum. An alternating piston pump was used for constant delivery of substrate solution and to achieve the desired residence time in the enzyme membrane reactor. The flow was regularly checked during the experiment (by measuring the volume of the product solution flowing from the reactor in a definite time). The reaction mixture was stirred on a magnetic stirrer. The enzyme membrane reactor was thermostated at 30 °C. The reaction solution that was pumped in the reactor was kept in a separate bottle on a magnetic stirrer and on ice to avoid substrate decomposition.

3. The mathematical model and data processing

The overall reaction rate of L-methionine oxidation catalyzed by L-PheDH was described using double-substrate Michaelis–Menten equation with competitive product inhibition (Eq. (1)). Since this is an equilibrium reaction, the kinetics of the reduction reaction was studied as well. The overall reaction rate of this reaction was described by three-substrate Michaelis–Menten equation with competitive inhibition by NAD^+ (Eq. (2)). The overall reaction rate of coenzyme regeneration catalyzed by NADH oxidase was described by Michaelis–Menten equation with anticompetitive NAD^+ inhibition (Eq. (3)). Mass balance equations for L-methionine oxidation in the batch reactor without coenzyme regeneration are presented by the Eqs. (4)–(8), and for the L-methionine oxidation with coenzyme regeneration Eqs. (4)–(6), (9) and (10).

Experiment of L-methionine oxidation with coenzyme regeneration was also carried out in the continuously operated enzyme membrane reactor. Mass balance equations for this reactor are presented by the Eqs. (11)–(15). Deactivation of the first order was incorporated in the mathematical model (for both enzymes: L-PheDH and NADH oxidase) for this experiment (Eq. (16))

$$r_1 = \frac{V_{m1} c_{\text{L-met}} c_{NAD^+} \gamma_{\text{L-PheDH}}}{(K_m^{\text{L-met}} + c_{\text{L-met}})(K_m^{NAD^+}(1 + c_{NADH}/K_i^{NADH}) + c_{NAD^+})} \quad (1)$$

$$r_2 = \frac{V_{m_2} c_{2-\text{oxo}} c_{NADH} c_{NH_4^+} \gamma_{\text{L-PheDH}}}{(K_m^{2-\text{oxo}} + c_{2-\text{oxo}})(K_m^{NADH}(1 + (c_{NAD^+}/K_i^{NAD^+})) + c_{NADH})(K_m^{NH_4^+} + c_{NH_4^+})} \quad (2)$$

$$r_3 = \frac{V_{m_3} c_{NADH} \gamma_{NOX}}{K^{NADH} + c_{NADH}(1 + (c_{NAD^+}/K_{i_2}^{NAD^+}))} \quad (3)$$

$$\frac{dc_{\text{L-met}}}{dt} = -r_1 + r_2 \quad (4)$$

$$\frac{dc_{2-\text{oxo}}}{dt} = r_1 - r_2 \quad (5)$$

$$\frac{dc_{NH_4^+}}{dt} = r_1 - r_2 \quad (6)$$

$$\frac{dc_{NAD^+}}{dt} = -r_1 + r_2 \quad (7)$$

$$\frac{dc_{NADH}}{dt} = r_1 - r_2 \quad (8)$$

$$\frac{dc_{NAD^+}}{dt} = -r_1 + r_2 + r_3 \quad (9)$$

$$\frac{dc_{NADH}}{dt} = r_1 - r_2 - r_3 \quad (10)$$

$$\frac{dc_{\text{L-met}}}{dt} = \frac{c_{\text{L-met0}} - c_{\text{L-met}}}{\tau} - r_1 + r_2 \quad (11)$$

$$\frac{dc_{2-\text{oxo}}}{dt} = \frac{c_{\text{2-oxo0}} - c_{\text{2-oxo}}}{\tau} + r_1 - r_2 \quad (12)$$

$$\frac{dc_{NH_4^+}}{dt} = \frac{c_{NH_4^+0} - c_{NH_4^+}}{\tau} + r_1 - r_2 \quad (13)$$

$$\frac{dc_{NAD^+}}{dt} = \frac{c_{NAD^+0} - c_{NAD^+}}{\tau} - r_1 + r_2 + r_3 \quad (14)$$

$$\frac{dc_{NADH}}{dt} = \frac{c_{NADH0} - c_{NADH}}{\tau} + r_1 - r_2 - r_3 \quad (15)$$

$$V_m = V_{m_0} e^{-k_d t} \quad (16)$$

Parameters of the mathematical model were estimated by non-linear regression analysis using Simplex and Least Squares method implemented in SCIENTIST software [33]. They were estimated by fitting the model to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine and fitted again until a minimal error between experimental and integrated values was achieved. The residual was defined as the sum of the squares of the differences between the experimental and calculated data. It was the "Episode" algorithm for a stiff system of differential equations, implemented in the SCIENTIST software that was used for simulations.

4. Results and discussion

4.1. pH optimization

Dependence of enzyme activity on pH was measured in different buffers for both enzymes (Fig. 2). It was found that NADH oxidase exhibits the highest activity at approximately pH 5.5. L-Phenylalanine dehydrogenase was found to be the most active around pH 10.0. Since it was obvious that sufficient activity of both enzymes is necessary

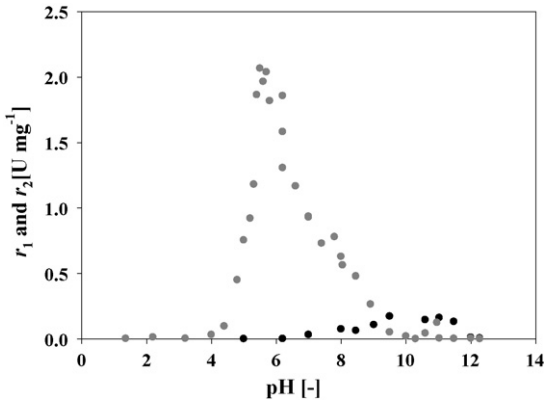

Fig. 2. pH optimization (30 °C), (●) L-PheDH from *Rhodococcus* sp. ($c_{\text{L-met}} = 0.5$ mmol dm^{-3}, $c_{\text{NAD}^+} = 0.5$ mmol dm^{-3}, $\gamma_{\text{L-PheDH}} = 0.086$ mg cm^{-3}), (●) NADH oxidase from *L. brevis* ($c_{\text{NADH}} = 0.2$ mmol dm^{-3}, $\gamma_{\text{NADH oxidase}} = 0.0042$ mg cm^{-3}).

Table 1
Kinetic parameters estimated from the initial reaction rate experiments

Parameter	Unit	Value
L-methionine oxidation		
V_{m_1}	U mg^{-1}	0.949 ± 0.028
$K_m^{\text{L-met}}$	mmol dm^{-3}	2.709 ± 0.349
$K_m^{\text{NAD+}}$	mmol dm^{-3}	1.310 ± 0.164
K_i^{NADH}	mmol dm^{-3}	0.034 ± 0.013
2-oxo-4-methylthiobutyric acid reduction		
V_{m_2}	U mg^{-1}	47.240 ± 1.660
$K_m^{2-\text{okso}}$	mmol dm^{-3}	6.153 ± 0.802
K_m^{NADH}	mmol dm^{-3}	0.029 ± 0.0027
$K_m^{NH_4^+}$	mmol dm^{-3}	133.120 ± 11.549
$K_i^{\text{NAD}^+}$	mmol dm^{-3}	0.031 ± 0.011
NAD$^+$ regeneration		
V_{m_3}	U mg^{-1}	3.408 ± 0.120
$K_{m_2}^{\text{NADH}}$	mmol dm^{-3}	0.247 ± 0.016
$K_{i_2}^{\text{NAD}^+}$	mmol dm^{-3}	0.134 ± 0.018

in order for the regeneration to work, a compromise had been made. L-PheDH activity is of crucial concern for L-methionine oxidation. NADH oxidase is much more active and more specific to its substrate than L-PheDH is towards L-methionine. L-PheDH exhibits an optimum activity for the reverse reaction (2-oxo-4-methylthiobutyric acid reduction) at pH 8.0 [34]. Considering that, a compromise has been found at pH 9.0. At this pH both enzymes are still active enough to catalyze the L-methionine oxidation with coenzyme regeneration, and the reverse reaction (α-keto acid reduction) is not favored.

4.2. L-*Phenylalanine dehydrogenase kinetics*

L-PheDH kinetics was measured by the initial reaction rate method. Since this enzyme catalyzes an equilibrium reaction, it was necessary to study both the oxidation of L-methionine and the reduction of 2-oxo-4-methylthiobutyric acid. The influence of all reaction compounds on the initial reaction rate was measured. Overall kinetic results are presented in Figs. 3 and 4, and parameters were estimated using double-substrate (L-methionine oxidation, Eq. (1)) or three-substrate (2-oxo-4-methylthiobutyric acid reduction, Eq. (2))

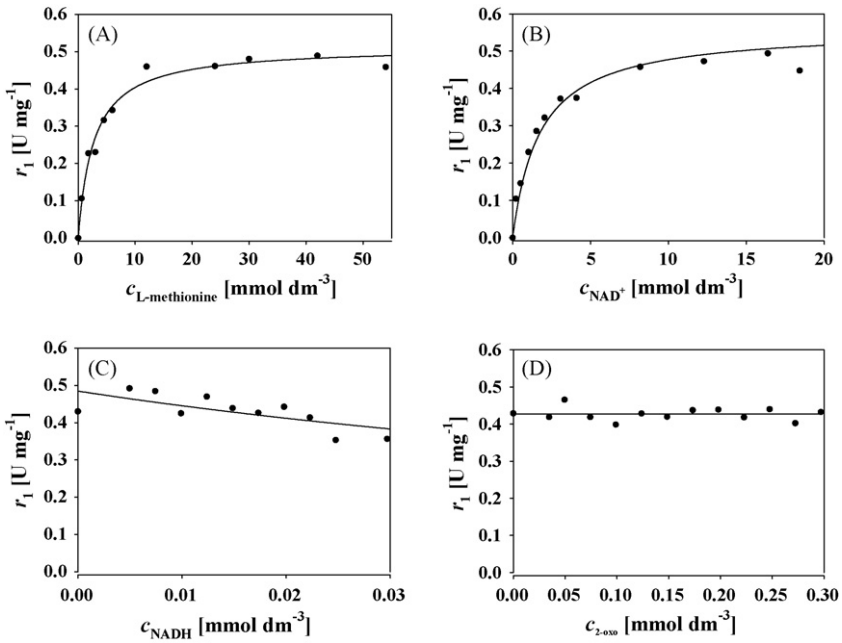

Fig. 3. L-PheDH kinetics in the reaction of L-methionine oxidation (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $\gamma_{\text{L-PheDH}} = 0.086$ mg cm^{-3}). Dependence of initial reaction rate on the concentration of: (A) L-methionine ($c_{\text{NAD}^+} = 2.05$ mmol dm^{-3}), (B) NAD$^+$ ($c_{\text{L-met}} = 5.94$ mmol dm^{-3}), (C) NADH ($c_{\text{NAD}^+} = 2.05$ mmol dm^{-3}, $c_{\text{L-met}} = 5.94$ mmol dm^{-3}), and (D) 2-oxo-4-methylthiobutyric acid ($c_{\text{NAD}^+} = 2.05$ mmol dm^{-3}, $c_{\text{L-met}} = 5.94$ mmol dm^{-3}).

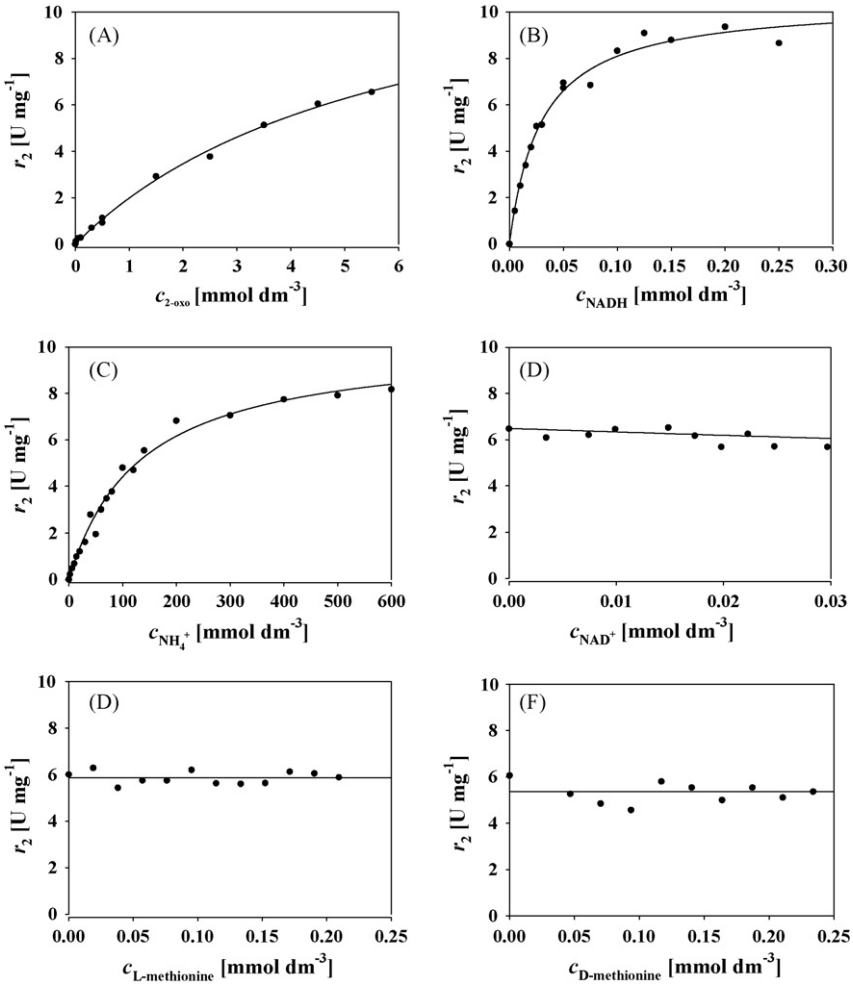

Fig. 4. L-PheDH kinetics in the reaction of 2-oxo-4-methylthiobutyric acid reduction (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $\gamma_{L\text{-PheDH}} = 0.086$ mg cm^{-3}). Dependence of initial reaction rate on the concentration of: (A) 2-oxo-4-methylthiobutyric acid ($c_{NADH} = 0.05$ mmol dm^{-3}, $c_{NH_4^+} = 198.02$ mmol dm^{-3}), (B) NADH ($c_{2\text{-oxo}} = 3.10$ mmol dm^{-3}, $c_{NH_4^+} = 198.02$ mmol dm^{-3}), (C) NH_4^+ ($c_{2\text{-oxo}} = 3.10$ mmol dm^{-3}, $c_{NADH} = 0.05$ mmol dm^{-3}), (D) NAD^+ ($c_{2\text{-oxo}} = 3.10$ mmol dm^{-3}, $c_{NH_4^+} = 198.02$ mmol dm^{-3}, $c_{NADH} = 0.25$ mmol dm^{-3}), (E) L-methionine ($c_{2\text{-oxo}} = 3.10$ mmol dm^{-3}, $c_{NH_4^+} = 198.02$ mmol dm^{-3}, $c_{NADH} = 0.25$ mmol dm^{-3}), and (F) D-methionine ($c_{2\text{-oxo}} = 3.10$ mmol dm^{-3}, $c_{NH_4^+} = 198.02$ mmol dm^{-3}, $c_{NADH} = 0.25$ mmol dm^{-3}).

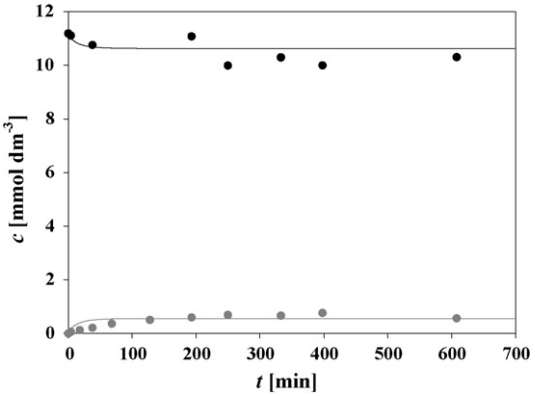

Fig. 5. L-Methionine oxidation without NAD^+ regeneration in the batch reactor (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $\gamma_{L\text{-PheDH}} = 0.086$ mg cm^{-3}, $c_{L\text{-met}} = 11.19$ mmol dm^{-3}, $c_{NAD^+} = 10.00$ mmol dm^{-3}, (●) L-methionine concentration, (●) 2-oxo-4-methylthiobutyric acid, (—) L-methionine model, (▬) 2-oxo-4-methylthiobutyric acid).

Table 2
Enzyme deactivation constants estimated from the batch reactor without coenzyme regeneration and the continuously operated enzyme membrane reactor

Parameter	Unit	Value
L-Methionine oxidation without coenzyme regeneration in the batch reactor		
k_d (L-PheDH)	min^{-1}	0.05180 ± 0.00890
L-Methionine oxidation with coenzyme regeneration in the continuous enzyme membrane reactor		
0–4 h		
k_d (L-PheDH)	min^{-1}	0.00447 ± 0.00038
k_d (NOX)	min^{-1}	0.13594 ± 0.00852
After the addition of fresh NOX		
k_d (L-PheDH)	min^{-1}	0.00083 ± 0.00008
k_d (NOX)	min^{-1}	0.01250 ± 0.00093

Michaelis–Menten kinetics. It was found that NADH inhibits the enzyme (Fig. 3C) in the reaction of L-methionine oxidation, while NAD^+ inhibits the enzyme in the reaction of 2-oxo-4-methylthiobutyric acid reduction (Fig. 4D). The developed mathematical model for L-methionine oxidation without coenzyme regeneration in the batch reactor was presented (Kinetic model: 1 and 2, Reactor model: 4–8). Estimated kinetic parameters are presented in Table 1.

4.3. L-Methionine oxidation without coenzyme regeneration in the batch reactor—mathematical model validation

L-Methionine oxidation without NAD^+ regeneration was carried out in the batch reactor (Fig. 5) to validate the developed mathematical model (Kinetic model: 1 and 2, Reactor model: 4–8). It was found that only 5% of L-methionine conversion could be achieved. The position of the reaction equilibrium was unfavorable. Therefore, it was necessary to shift it towards the wanted product—2-oxo-4-methylthiobutyric acid. The developed mathematical model described the data well. It was necessary to consider L-PheDH deactivation which occurred in the experiment. Enzyme deactivation constant of the first order was estimated and is presented in Table 2.

4.4. NADH oxidase kinetics

The results of the influence of all reaction compounds on the initial reaction rate of NADH oxidase catalyzed NADH oxidation was presented in Fig. 6. This includes both the reaction product NAD^+ (Fig. 6B), and other compounds like L-methionine (Fig. 6C), 2-oxo-4-methylthiobutyric acid (Fig. 6D) and D-methionine (Fig. 6E). It was found that NAD^+ acts as anticompetitive inhibitor of the enzyme (Fig. 6B). Estimated kinetic parameters which concern NADH oxidase are presented in Table 1. Since oxygen concentration in all cases was constant at around 0.25 mmol dm^{-3}, this is an apparent NADH oxidase kinetics.

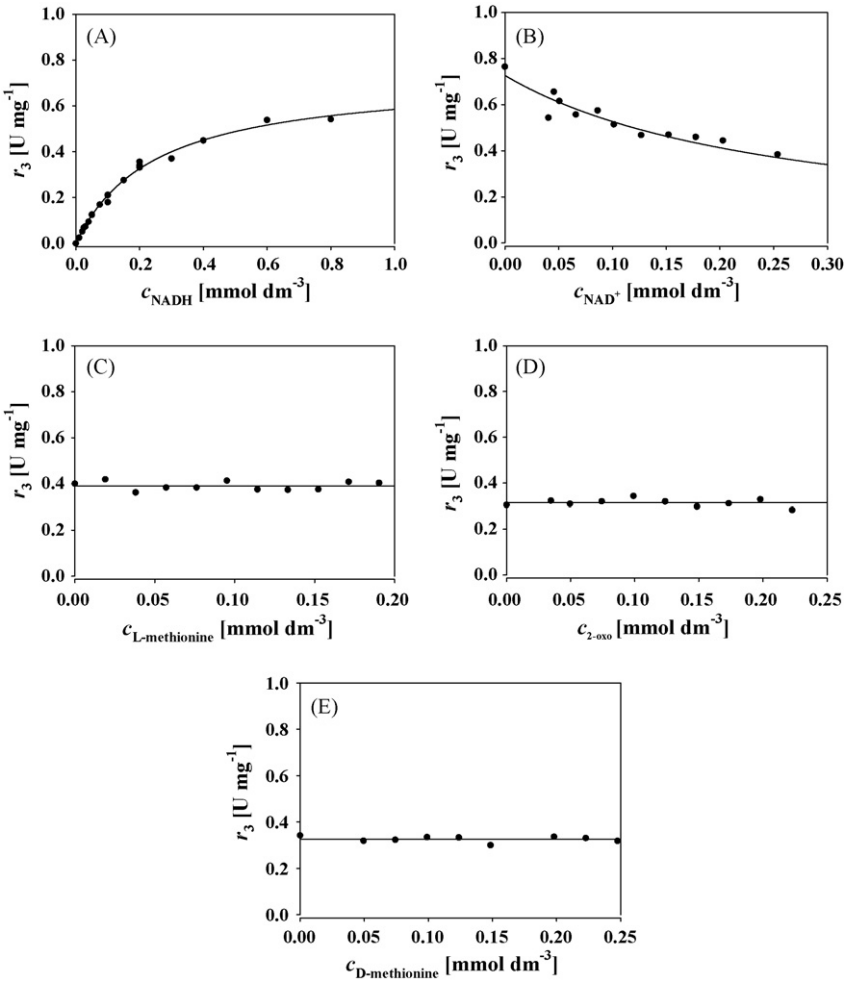

Fig. 6. NADH oxidase apparent kinetics in the reaction of NAD^+ regeneration (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $\gamma_{NADH\ oxidase} = 2.0$ mg cm^{-3}). Dependence of initial reaction rate on the concentration of: (A) NADH, (B) NAD^+ ($c_{NADH} = 0.026$ mmol dm^{-3}), (C) L-methionine ($c_{NADH} = 1.00$ mmol dm^{-3}), (D) 2-oxo-4-methylthiobutyric acid ($c_{NADH} = 1.00$ mmol dm^{-3}), and (E) D-methionine ($c_{NADH} = 1.00$ mmol dm^{-3}).

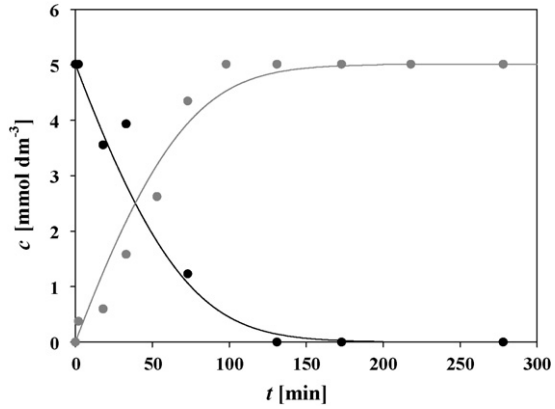

Fig. 7. L-Methionine oxidation with NAD^+ regeneration by NADH oxidase from *L. brevis* in the batch reactor (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $c_{L\text{-met}} = 5$ mmol dm^{-3}, $c_{NAD^+} = 0.2$ mmol dm^{-3}, $c_{L\text{-PheDH}} = 0.97$ mg cm^{-3}, $\gamma_{NADH\ oxidase} = 2.3$ mg cm^{-3}, (●) L-methionine concentration, (●) 2-oxo-4-methylthiobutyric acid, (—) L-methionine model, (—) 2-oxo-4-methylthiobutyric acid).

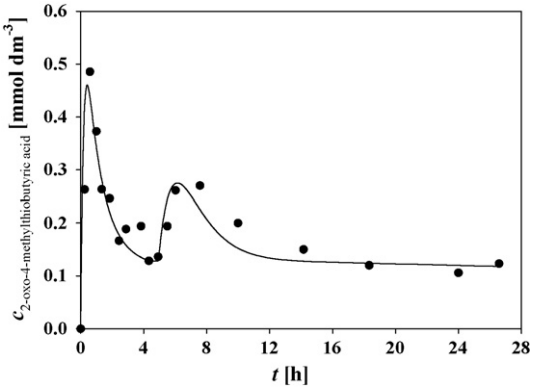

Fig. 8. L-Methionine oxidation with NAD^+ regeneration by NADH oxidase from *L. brevis* in the continuously operated enzyme membrane reactor (30 °C, pH 9.0, 0.2 mol dm^{-3} Tris–HCl buffer, $c_{L\text{-met}} = 5$ mmol dm^{-3}, $c_{NAD^+} = 0.2$ mmol dm^{-3}, $\gamma_{L\text{-PheDH}} = 2.0$ mg cm^{-3}, $\gamma_{NADH\ oxidase} = 2.333$ mg cm^{-3}, $\tau = 60$ min, (●) 2-oxo-4-methylthiobutyric acid concentration, (—) 2-oxo-4-methylthiobutyric acid model).

4.5. L-Methionine oxidation with coenzyme regeneration by NADH oxidase from L. brevis in the batch reactor—mathematical model validation

L-Methionine oxidation with coenzyme regeneration catalyzed by NADH oxidase from *L. brevis* was carried out in the batch reactor (Fig. 7). NADH oxidase was used to remove the NADH from the reaction system and to convert it to the needed NAD^+. This enables the use of lower concentration of expensive coenzyme, and the shifting of the reaction equilibrium towards the creation of 2-oxo-4-methylthiobutyric acid. L-Methionine conversion was 100% in this experiment (Fig. 7). The developed mathematical model (Kinetic model: Eqs. (1)–(3), Batch reactor model: Eqs. (4)–(6), (9) and (10)) simulated the experiment well. NAD^+ regeneration by NADH oxidase was successful.

4.6. L-Methionine oxidation with coenzyme regeneration by NADH oxidase from L. brevis in the continuously operated enzyme membrane reactor—mathematical model validation

The results of L-methionine oxidation with coenzyme regeneration carried out in the continuously operated enzyme membrane reactor are shown in Fig. 8. Both enzymes (L-PheDH and NADH oxidase) were added in the reactor through the injection septum. L-Methionine conversion was low in this experiment. The highest conversion was achieved at approximately 35 min of experiment and it was only 9%. After that time L-methionine conversion rapidly dropped to its lowest value of 2.2%. A fresh NADH oxidase was added due to the assumption that it had been deactivated. This assumption was checked by the additional experiments presented elsewhere [35]. L-Methionine conversion increased after the NADH oxidase addition but not significantly (up to 5%) and dropped again. A steady state was achieved at earlier mentioned 2.2% of L-methionine conversion. It was assumed that NADH oxidase deactivation occurred. Enzyme deactivation constant of the first order was estimated and presented in Table 2. Prior reports on NADH oxidase from *L. brevis* indicate its quick deactivation in the presence of oxygen due to the cystein on its active site [27]. This was confirmed for NADH oxidases from other sources as well [15,36]. Cystein and similar redox-active thiols in the active site of an NADH oxidase make these enzymes turnover limited [16,37] which means that they have lower operational stability [10].

As NADH oxidase activity depends on oxygen concentration, its lower concentration means lower NADH oxidase activity (since oxygen concentration is the limiting factor). In that case NADH oxidase activity might have been too low and the regeneration reaction too slow. As the consequence L-methionine conversion was just a little higher than in the batch reactor without coenzyme regeneration. Besides NADH oxidase, it was assumed that L-phenylalanine dehydrogenase deactivates as well, as it was the case in the first batch reactor experiment. The deactivation constant of L-PheDH was also estimated and it was found that its value is about 30-fold lower than it was in the case of NADH oxidase (Table 2). L-PheDH deactivation constant is even lower (fivefold) after the addition of fresh NADH oxidase in the reactor, which could be explained by enzyme stabilization. The extended mathematical model (Kinetic model: Eqs. (1)–(3) and Eq. (16), continuously operated enzyme membrane reactor model: Eqs. (11)–(15)) which includes deactivation of enzymes described the data well. Even though NADH oxidase deactivation was complete, that appeared not to be the case for L-phenylalanine dehydrogenase. L-Methionine conversion of approximately 2.2% was constant through the longer period of time, like in the batch experiment without coenzyme regeneration (Fig. 5). The estimated values of deactivation constants for L-phenylalanine dehydrogenase are much lower than in the case of NADH oxidase which confirms its faster deactivation.

5. Conclusions

L-Phenylalanine dehydrogenase from *Rhodococcus* sp. M4 was able to convert L-methionine to 2-oxo-4-methylthiobutyric acid in the batch system but with low L-methionine conversion of only 5%. The enzyme is inhibited by NADH as a

product of L-methionine oxidation and by NAD^+ as a product of reverse reaction—2-oxo-4-methylthiobutyric acid reduction. NADH oxidase from *L. brevis* was successfully used as the catalyst in the coenzyme regeneration system. It was found that it is inhibited only by NAD^+, and not by other reaction compounds. Mathematical models of L-methionine oxidation without and with coenzyme regeneration in the batch reactor and continuously operated enzyme membrane reactor were developed and validated in reactor experiments. L-Methionine conversion of 100% was achieved in the batch system using NADH oxidase as the regeneration enzyme. Experiment in the enzyme membrane reactor showed deactivation of both enzymes which was quantified by the corresponding deactivation constants. The studied system shows good efficiency in converting L-methionine in the batch reactor. Continuously operated enzyme membrane reactor does not represent a good reactor mode for the L-methionine oxidation catalyzed by these enzymes. Since the final product concentration in the continuously operated reactor is low, final yield after the separation and purification step would be low. As for the batch reactor is concerned, final yield after the separation and product purification would be much higher because there is no reactant left in the reaction solution.

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