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Development of NAD$^+$ Regeneration Process in Microreactors of Different Materials

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Coenzyme NAD$^+$ (nicotinamide adenine dinucleotide) is used in numerous biocatalytic oxidation reactions where NAD$^+$ is reduced to NADH (reduced form of nicotinamide adenine dinucleotide). As the price of coenzyme NAD$^+$ is extremely high, it is essential to regenerate the reduced form of coenzyme back into the oxidized form. In this study regeneration of coenzyme NAD$^+$ was carried out by reversible oxidation of ethanol to acetaldehyde using different biocatalysts and microreactors of different materials. Based on experimental results, by measuring dependence of the reaction rate on the concentration of all components of the reaction system, kinetics of coenzyme regeneration was estimated and the mathematical model of the process was developed. The enzyme kinetics was modeled as a pseudo-homogeneous process with the double substrate Michaelis–Menten rate expression. Validation of developed mathematical model of the process was carried out on a series of independent experiments. Based on results and good agreement between model predictions and experimental results, obtained model simulation results could be used for further process optimization and development of new microreactors. Additionally, comparison of NAD$^+$ coenzyme regeneration within a microreactor and in a batch reactor using immobilized enzyme and enzyme suspension was made. The best results were noticed when glass microreactor with suspended ADH was used (X = 95.89 %; τ = 2 s).

1. Introduction

In the field of biotransformation NAD(P)-dependent oxidoreductases represent a great interest because they are at the crossroads of numerous syntheses of high added value compounds in pharmaceutical, food, cosmetic and agrochemical industry. Their main disadvantage is that they are dependent on the presence of cofactors, NAD(P)$^+$ or NAD(P)H, in the reaction system. For maximal efficiency of those reactions coenzymes should be added in a stoichiometric amount and may not be replaced by more economical synthetic products so an efficient and economical coenzyme regeneration system is necessary. Because its oxidation state is changing during the reaction, continuous regeneration is necessary and it represents an important industrial challenge (Theodore et al., 2010).
Up to now many *in situ* methods for cofactor regeneration have been studied and developed. These methods can be divided into several categories: chemical, biological, electrochemical, photochemical and enzymatic (Findrik et al., 2007). Among them, the enzymatic methods seem to be the most conventional and useful.

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Regeneration can be implemented not only in the conventional macroreactor systems, but also in the reactor system, called microreactor, whose dimensions are order of magnitude of ten nanoliters to one milliliter. Microreactors consist of microchannels that are extremely small dimensions, characteristic sizes of 10 – 500 μm, carved into tiles of glass, silicon, silica, polymers and other materials (Ehrfeld et al., 2000). Large surface to volume ratio, excellent mass and heath transfer, shorter residence time, smaller amount of reagents, catalyst and waste products comparing to equivalent bulk reactions, laminar flow, effective mixing and better process control, small energy consumption and less environmental impact are just some of the microsystem advantages (Žnidaršič-Plazl and Plazl, 2009).
In this study implementation of microreactor technology and enzymatic methods for coenzyme regeneration are investigated in microchannels of different materials, e.g. glass and polytetrafluoroethylene (PTFE). Suspended and immobilized enzyme alcohol dehydrogenase (ADH) isolated from baker yeast and suspended and immobilized permeabilized yeast cells were used for the NAD^+ regeneration in the reaction of acetaldehyde reduction to ethanol. Conversion of approximately 100 % for residence time shorter than 2 s was observed when suspended ADH was used. For all investigated regeneration systems reaction kinetics was measured and mathematical model was developed. Finally, the model prediction results were proven and verified on a set of independent experiments performed in a microreactor. Based on results and good agreement between model predictions and experimental results, obtained model simulation results could be used for further process optimization and development of new microreactor systems.

2. Materials and methods

2.1 Methods

Yeast cell's permeabilization was carried out according to the procedure described by Vrsalović Presečki and Vasić-Rački (2005) and biocatalyst immobilization was carried out according to the procedure described by Stojkovič and Žnidaršič-Plazl (2010).

2.2 Kinetic parameter estimation

Regeneration process was carried out as a two-substrate reaction of acetaldehyde and the coenzyme NADH with enzyme ADH dissolved in a aqueous buffer (75 mmol/dm^3 glycine-pyrophosphate buffer, pH = 9, T = 25 °C) in the microreactor (length: width: depth = 330 mm : 250: 50 μm with the internal volume of 6 mm^3; Figure 1). Enzyme and coenzyme dissolved in aqueous buffer were fed in microreactor from one inflow, while the substrate (acetaldehyde or ethanol) was fed from another inflow using two syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, USA) equipped with high pressure stainless steel syringes (8 cm^3, Harvard Apparatus, USA). Concentration of substrate and coenzyme was altered, while the biocatalyst concentration (γ_{ADH} = 0.2 g/dm^3) was kept constant in all performed experiments. The solutions were fed at a 1:1 volumetric flow ratio and the parallel flow of two liquids was established.

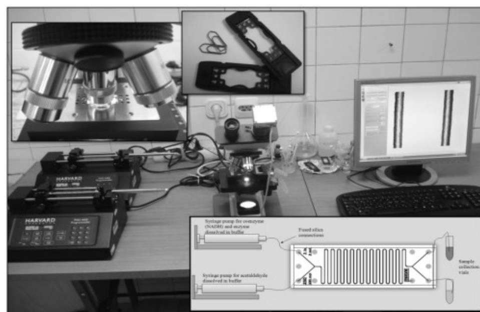

Figure1. Experimental set-up used to perform NAD^+ regeneration in the microreactor

2.3 NAD^+ coenzyme regeneration in different reaction systems

After kinetic parameters were estimated, the possibility to use dissolved and immobilized enzyme alcohol dehydrogenase and permeabilized yeast cells within microchannel of different materials (glass microchannel with inner diameter of width:depth = 50:250 μm and polytetrafluoroethylene (PTFE) with

inner diameter width:depth = 1000:1000 µm) was explored. Biocatalyst and coenzyme were dissolved in aqueous buffer (75 mmol/dm^3 glycine-pyrophosphate buffer, pH = 9, T = 25 °C) in experiments performed with dissolved enzyme and yeast cells. They were fed from one inflow, while the substrate (acetaldehyde dissolved in buffer; 75 mmol/ dm^3 glycine-pyrophosphate buffer, pH = 9, T = 25 °C) was fed from other inlet. The inlet concentration of acetaldehyde and NADH were kept constant during all experiments ($c_{i,NADH}$ = 5.5 mmol/dm^3, $c_{i,acetaldehyde}$ = 5.5 mmol/dm^3) while the inlet volume activity of permeabilized cells and enzyme was set to be approximately 60 U/cm^3. The fluid flow profiles in the glass microchannel were monitored under the microscope. In experiments performed with immobilized enzyme and yeast cells in both types of microreactor, inlet concentrations of coenzyme and hexanol were the same as in previous experiments ($c_{i,NADH}$ = 5.5 mmol/dm^3, $c_{i,acetaldehyde}$ = 5.5 mmol/dm^3) while the concentration/volume activity of biocatalyst was influenced by the immobilization efficiency. Outflows from the microreactors containing the substrate, product, biocatalyst, NADH and NAD$^+$ were collected in vials placed on ice to stop the reaction via enzyme inactivation. The samples were collected and analyzed.

2.4 Analytics

Ethanol concentration was determinated using GC (Shimadzu GC-2014, Kyoto, Japan) with the flame ionization detector. Polar column ZB-WAX (Phenomenex, Torrance, USA) and helium as gas carrier were used. Samples were prepared by mixing the equal volume of sample with internal standard (1 % solution of acetonitrile) for 1 min. Before the injection (1 mm^3) samples were centrifuged (Hettich, Universal 320R, Andreas Hettich GmbH & Co. KG, Germany) for 3 min at 4 °C and 9000 min^{-1} and filtrated (Filter Chromafil® AO-20/3; 0.2 µm, 3 mmo100, Macherey, Nagel GmbH, Germany).
Determination of NADH concentration was performed before and during all experiments by the spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at λ = 340 nm.

3. Results and discussion

3.1 Parameter estimation and model validation

A process of NAD$^+$ regeneration in microreactor was developed and the kinetics parameters of enzymatic reactions were estimated. The developed mathematical model of the process was comprised of kinetic model and mass balances for reactants and products (Table 1). Two-substrate enzyme kinetics constitutes most of all enzyme reactions, and other substrate is mostly coenzyme. In reactions of biotransformation often comes to inhibition and deactivation of enzyme. Kinetic model for this reaction is described by Michaelis-Menten equation for two-substrate reaction with competitive inhibition of the product (Table 1).

Table 1. Kinetic and mass balance equations of the coenzyme regeneration in microreactor

Scheme of biocatalytic acetaldehyde reduction by isolated ADH	
$C_2H_5OH \underset{r_2}{\overset{r_1}{\rightleftarrows}} CH_3CHO$ (ADH; NAD^+ / $NADH + H^+$)	
Kinetic model	**Mass balances**
$r_1 = \dfrac{V_{m1} \times c_{acetaldehyde} \times c_{NADH} \times \gamma_{ADH}}{(K_m^{acetaldehyde} + c_{acetaldehyde}) \times (K_m^{NADH} + c_{NADH})}$	$\dfrac{dc_{acetaldehyde}}{dx} = -\dfrac{1}{v} \times r_1 + \dfrac{1}{v} \times r_2$
	$\dfrac{dc_{NADH}}{dx} = -\dfrac{1}{v} \times r_1 + \dfrac{1}{v} \times r_2$
$r_2 = \dfrac{V_{m2} \times c_{ethanol} \times c_{NAD^+} \times \gamma_{ADH}}{(K_m^{ethanol} \times (1 + \dfrac{c_{acetaldehyde}}{K_i^{acetaldehyde}}) + c_{ethanol}) \times (K_m^{NAD^+} + c_{NAD^+})}$	$\dfrac{dc_{ethanol}}{dx} = \dfrac{1}{v} \times r_1 - \dfrac{1}{v} \times r_2$
	$\dfrac{dc_{NAD^+}}{dx} = \dfrac{1}{v} \times r_1 - \dfrac{1}{v} \times r_2$

Reaction rates were measured for residence time of τ = 3.6 s and reactor content was considered to be homogenous. A model of steady state parallel flow reactor with negligible axial dispersion was used for description of coenzyme regeneration in the microreactors. It was assumed that there were no radial variations in velocity, concentrations or reaction rates. Mass balances for the acetaldehyde reduction are based on assumption of constant reaction volume. Additionally, to simplify the model, the change in the mass transfer coefficient along the reactor was assumed to be negligible. Mass balances for ADH catalyzed reaction in microreactor are given by equations for acetaldehyde, NADH, ethanol and NAD^+ (Table 1) were x is microreactor length, v linear velocity and r reaction rate in U/mg with the boundary condition $c(x=0)=c_i$.

Kinetics of the reaction of reduction and oxidation were investigated by measuring the initial reaction rate dependence on reactant and product concentrations. Because of the absences of product inhibition, the parameters for acetaldehyde reduction were estimated by nonlinear regression using experimental results and double substrate Michaelis-Menten kinetics (Table 2). From the estimated constants for the acetaldehyde reduction it can be seen that maximal initial rate (622.44 ± 67.17 U/mg) is significantly higher than the one estimated from cuvette measurements (171.074 ± 3.801 U/mg). These results confirm one of microreactor advantages in comparison to classical reactor systems, meaning fast mass transfer and high volume to surface area. For all the other parameters, some differences were noticed but not as significant as for maximal initial rate.

Table 2. Comparison of kinetic parameters estimated from the measurements of the independent initial reaction rates in microreactor and cuvette

	Microreacotor (V = 6mm^3)	**Cuvette** (V = 1 cm^3) (Vrsalović Presečki, 2006)
	Acetaldehyde reduction	
V_m [U/mg]	622.44 ± 67.17	171.074 ± 3.801
$K_m^{acetaldeyded}$ [mmol/dm^3]	0.568 ± 0.142	0.584 ± 0.071
K_m^{NADH} [mmol/dm^3]	2.509± 0.941	0.152 ± 0.006
$K_i^{ethanol}$ [mmol/dm^3]	-	1.298 ± 0.078
K_i^{NAD+} [mmol/dm^3]	-	0.877 ± 0.069
	Ethanol oxidation	
V_m [U/mg s]	216.42 ± 39.01	144.580 ± 2.520
$K_m^{ethanol}$ [mmol/dm^3]	37.398 ± 8.142	8.271 ± 0.613
K_m^{NAD+} [mmol/dm^3]	3.703 ± 1.443	0.333 ± 0.025
$K_i^{acetaldehyde}$ [mmol/dm^3]	0.32205 ± 0.0628	0.064 ± 0.006
K_i^{NADH} [mmol/dm^3]	-	0.029 ± 0.002

Kinetics of ethanol oxidation was also investigated by measuring the initial reaction rate dependence on concentrations of the reactants, ethanol and NAD^+, and products, acetaldehyde and NADH. It was observed, that the initial rate of ethanol oxidation was inhibited by acetaldehyde, while coenzyme inhibition was not noticed for investigated concentration range. The parameters for ethanol oxidation (Table 2) were estimated by nonlinear regression using experimental results and double substrate Michaelis-Menten kinetics with acetaldehyde inhibition (Table 1).

In order to validate the developed mathematical model of coenzyme NAD^+ regeneration in microreactor independent experiment was performed ($c_{i,\mathrm{NADH}}$ = 6.9 mmol/dm^3, $\gamma_{i,\mathrm{ADH}}$ = 0.2 g/dm^3, $c_{i,\mathrm{acetaldehyde}}$ = 5.5 mmol/dm^3, 75 mmol/dm^3 glycine-pyrophosphate buffer, pH = 9; T = 25 °C). As shown in Figure 2 the developed model describes experimental data very well indicating that it could be used for further process optimisation and scale-up (numbering-up) of microreactors. Under described conditions conversion of approximately 80 % could be achieved. For the model simulations with higher initial concentration of acetaldehyde ($c_{\mathrm{i,NADH}}$ = 5.5 mmol/dm^3, $\gamma_{\mathrm{i,ADH}}$ = 0.2 g/dm^3, $c_{\mathrm{i,acetaldehyde}}$ = 44 mmol/dm^3, 75 mmol/dm^3 glycine-pyrophosphate buffer, pH = 9; T = 25 °C) conversion of NADH was predicted to be 100 % (Figure 2).

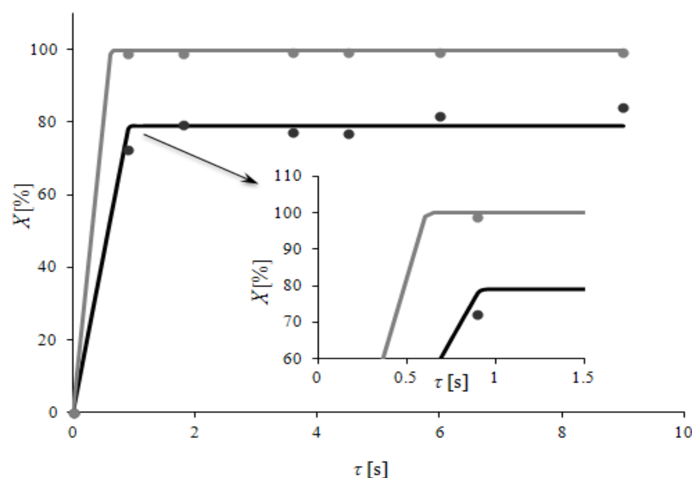

Figure 2. NAD$^+$ regeneration in microreactor ($\gamma_{i,ADH}$ = 0.2 g/dm^3, 75 mmol/dm^3 glycine-pyrophosphate buffer, pH = 9, T = 25 °C) [line: model, ●: $c_{i,NADH}$ = 6.9 mmol/dm^3, $c_{i,acetaldehyde}$ = 5.5 mmol/dm^3 experiment; ●: $c_{i,NADH}$ 5.5 mmol/dm^3, $c_{i,acetaldehyde}$ = 44 mmol/dm^3 experiment]

3.2 Comparison of different systems for NAD$^+$ coenzyme regeneration

Suspended and immobilized enzyme ADH isolated from baker yeast and suspended and immobilized permeabilized yeast cells (Figure 3a and 3b) were used for the NAD$^+$ regeneration process. Additionally, two microchannels of different origins, e.g. glass and polytetrafluoroethylene (PTFE) were tested in order to determine which system would provide best results, e.g. highest productivity and conversion, when coenzyme regeneration is considered.

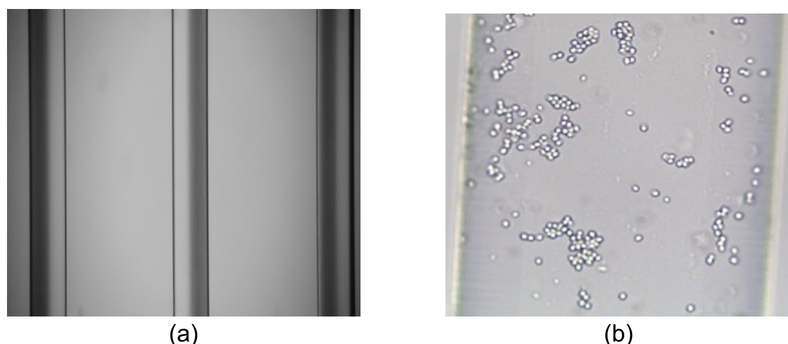

Figure 3. a) Parallel flow in glass microreactor at 50 mm^3/min (aqueous phase with acetaldehyde-white, aqueous phase with suspended yeast cells-grey) b) Saccharomyces cerevisiae cells immobilised into glass microchannel

Comparison of NAD$^+$ coenzyme regeneration was performed according to maximal conversion obtained for different types of biocatalyst (permeabilized yeast cells and enzyme, suspended and immobilized) and different types of reactors (microreactor (glass and PTFE) and a batch reactor) for the same inlet conditions (T = 25 °C, $c_{i,acetaldehyde}$= 5.5 mmol/dm^3, $c_{i,NADH}$ = 5.5 mmol/dm^3). Analysis of the results (Table 3) revealed that highest conversion (X = 95.89 %) of NADH to NAD$^+$ in shortest time (τ = 2 s) was achieved in glass microreactor when suspended enzyme was used. Comparing those results with the batch reactor in which the suspended isolated enzyme (92.73 %) and suspended permeabilized cells (91.38 %) were used, the conversion was slightly higher in microreactor. On the other hand comparing the time that was necessary for those conversions to be obtained, results are in favour of microreactor system.

Table 3. Comparison of NAD^+ coenzyme regeneration within a microreactor and a batch reactor using immobilized enzyme and enzyme suspension

	Type of biocatalyst	Type of reactor	τ (s)	X (%)
Suspended biocatalyst	Enzyme	Batch reactor (t =40 s)	-	92.73
		Glass microreactor	2	95.89
		PTFE microreactor	47.10	94.36(±1.62)
	Permeabilized baker's yeast cells	Batch reactor (t = 20s)	-	91.36
		Glass microreactor	7.2	69.86(±1.12)
		PTFE microreactor	47.10	86.67(±1.83)
Immobilized biocatalyst	Enzyme	Glass microreactor	3.6	11.99(±2.29)
		PTFE microreactor	94.3	11.91(±1.33)
	Permeabilized baker's yeast cells	Glass microreactor	3.6	7.80(±3.55)
		PTFE microreactor	94.3	6.58(±1.56)

Conversions that were obtained with immobilized biocatalyst were lower in comparison to experiments where suspended biocatalyst was used. Reaction with immobilized enzyme is much more economical taking into consideration the costs of free enzyme solution that is necessary to feed in to the reaction system continuously, the obtained conversion and the stability of the process (t = 6 days).

4. Conclusion

A mathematical model for NAD^+ coenzyme regeneration in a microreactor was developed. Parameters of the kinetics model were estimated from the experimental results obtained in the microreactor. From the estimated constants it can be seen that maximal initial rate obtained from microreactor measurements is significantly higher than the one estimated from cuvette measurements. For all the other parameters, some differences were noticed but not as significant as for maximal initial rate.

Additionally, two microreactors of different origins combined with suspended and immobilized biocatalyst were tested and the results were compared to batch experiments to find most suitable process for coenzyme regeneration. Best results, meaning highest conversion obtained in shortest time, were noticed when glass microreactor with suspended ADH was used (X = 95.89 %; τ = 2 s).

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