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NAD$^+$ regeneration in a microreactor using permeabilized baker's yeast cells

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ABSTRACT

NAD(P)-dependent oxidoreductases represent a great interest in the field of biotechnology and biotransformation. Although they have many advantages, the biggest drawback and limitation of oxidoreductase usage is the price of the coenzymes. In order to solve this problem, many in situ methods for regeneration of coenzymes have been studied and developed. Unfortunately, although results indicate that those methods are suitable for regeneration procedure, most of the processes need additional optimization to make them more sustainable. As an alternative, microreactor technology could be used as a new technique for coenzyme regeneration processes due to many advantages.

In this study regeneration of coenzyme NAD$^+$ was carried out in a microreactor by acetaldehyde reduction to ethanol using enzyme alcohol dehydrogenase (ADH). Suspended and immobilized whole permeabilized baker's yeast cells were used as the source of the ADH enzyme. A 65.3% conversion of NADH was achieved with suspended permeabilized baker's yeast cells for a residence time of $\tau = 36$ s and equimolar concentration of substrates ($c_{i,NADH} = 5.5$ mmol/dm^3, $c_{i,acetaldehyde} = 5.5$ mmol/dm^3). When working with immobilized cells, conversion achieved for the same residence time was 10 fold lower. When permeabilized baker's yeast cells were used for coenzyme regeneration process was stabile for 6 days of continuous operation which makes this system a good alternative for coenzyme regeneration.

1. Introduction

In the last two decades, microreactor technology has seen exponential growth. Decreased channel dimensions of microreactors (on the order of 10–1000 μm) afford numerous advantages that lead to increased reaction efficiency. High surface to volume ratio, faster diffusion dominated transport, enhanced heat transfer and thus reduced energy demands, good process control, high throughput, usage of minimal (microlitres) of reagent volumes, etc., are some of the system advantages [1]. Due to the small dimensions, reactors were proved useful in a wide range of applications from requirement of an integral component within the framework of process miniaturization as well as catalytic screening and process intensification [2], various aspects of technological and biotechnological cases such as on-site and on-demand production of chemicals. [3]. Although a great majority of the reaction systems that are studied in microreactors are connected with chemical synthesis, biocatalysis in a microreactor was shown to be a promising alternative [4]. Great enzyme diversity which can either be found in nature or generated with evolutionary methods or rational design [5], mild operating conditions, high observed reaction rates, simple catalyst preparation, region- and stereo-specificity are just some of the advantages that make biocatalysis so interesting. In addition, the use of enzymes for catalysis compared to classical chemical catalysts is highly desired in food industrial processes because resultant products are classified as "natural" by food regulatory agencies [6]. Since the use of purified enzymes is often limited by their high cost, the application of partially purified enzymes and crude cell lysates was suggested as an alternative [7,8]. Additionally, in the recent years studies on the use of enzymes within the whole cells used in suspended or immobilized form, have gained considerable attention. Lower costs, due to the fact that catalyst extraction and purification is avoided, an opportunity to recycle the enzyme-containing cells is provided and the biotransforming enzyme may be stabilized in the intracellular environment, are some advantages over isolated enzymes [9]. Besides all advantages, there are also some drawbacks and limitations of whole cell biocatalysis such as mass transfer limitations of the substrate and the product through the cell membrane and the specific activity that is lower in comparison to the pure enzymes [10]. In order to overcome mass transfer limitations, different techniques of membrane permeabilization (the use of organic solvents, mechanical damage, electroporation) are implemented [11].

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List of symbols

c	concentration (mmol/dm^3)
D	diffusion coefficient (m^2/s)
k_b	Boltzman's constant
K_i	inhibition constant (mmol/dm^3)
K_m	Michelis–Menten constant (mmol/dm^3)
r	reaction rate (U/mg)
R_H	hydrodynamic radius of the cells (μm)
T	temperature (°C)
t	time (h)
V	reactor volume (mm^3)
V_m	maximal reaction rate (U/g)
V.A.	volumetric activity (U/cm^3)
X	conversion (%)
γ	mass concentration (g/cm^3)
λ	wavelength (nm)
η	dynamic viscosity (kg/(m s))
ρ	density (kg/m^3)
τ	residence time (s)
τ_D	diffusion time (s)
Φ	flow rate (mm^3/min)

Abbreviations

ADH	alcohol dehydrogenase
APTES	3-aminopropyltrietoxysilane
CTAB	hexadecyltrimethylammonium bromide
i	inlet
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrate

In order to overcome some disadvantages of suspended biocatalyst usage, like decrease of stability during storage, biocatalyst immobilization is often implemented in microreactors. Usually, the biocatalysts immobilized on the glass microchip are more stabile and more resistant to environmental changes in comparison to suspended biocatalysts and can be reused multiple times [12]. However, immobilization efficiency significantly varies depending on the method and expensive and time-consuming immobilization techniques are still some of immobilization implementation disadvantages [13].

In our previous work hexanol oxidation process by enzyme alcohol dehydrogenase as biocatalyst was developed. The results that were obtained indicated that microreactors could be better solution for hexanal production in comparison to traditional biotransformation in batch system (macroreactor) [14,15]. Although the system was sustainable, one of the biggest drawbacks and limitations of the developed system was the price of the coenzyme that is essential for ADH functionality since it must be added in a stoichiometric amount and may not be replaced by more economical synthetic products. In order to solve this problem preliminary investigations were performed and different regeneration and reactor systems were compared [16]. The implementation of microreactor technology and the enzymatic method for NAD^+ coenzyme regeneration were intensively investigated in a glass microreactor [17]. As a biocatalyst, pure enzyme alcohol dehydrogenase was used. It was stressed down how important is the process of successful coenzyme regeneration in order to make the coenzyme dependent process sustainable and economical. Obtained results (X = 95.89%; $c_{i,acetadahyde}$ = 5.5 mmol/dm^3, $c_{i,NADH}$ = 5.5 mmol/dm^3, $\gamma_{i,ADH}$ = 0.2 g/dm^3) indicated that microreactors could be a good alternative for biocatalytical coenzyme regeneration process.

In order to make the process of coenzyme regeneration even more economical possibility to use whole cells for the coenzyme regeneration was investigated in the present study. Suspended and immobilized permeabilized baker's yeast cells were used as a source of enzyme alcohol dehydrogenase (ADH) because of their price and availability. Acetaldehyde was used as a substrate for the coenzyme regeneration because of its low price and a high specificity of the ADH towards it [18–20]. Disadvantages of selected reaction system are discussed elsewhere [17]. Additionally, fluid flow of two aqueous phases in microchannel was monitored and influence of flow rates on distribution of two phases for different flows was described. The reaction kinetics of coenzyme regeneration in microreactor using suspended permeabilized yeast cells was measured and a previously proposed 2D mathematical model [17,21] containing convection, diffusion and enzyme reaction terms was used for process simulation. The model prediction results were proven and verified on a set of independent experiments performed in a microreactor. Influence of total flow rates on cells detachment were tested in the experiments performed using immobilized cells. Critical flow rate responsible for detachment of the immobilized cells was determined. Activity of the immobilized cells was monitored for long period of time at constant flow rate in order to determine the possibility of reuse of immobilized biocatalyst. Additionally, comparison between application of suspended and immobilized biocatalyst in process of coenzyme regeneration was performed. It was found that immobilized cells could be efficiently reused making the system sustainable in comparison to system with suspended cells.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

NAD^+ and NADH were purchased from Jülich Fine Chemicals (Germany). Acetaldehyde and hexadecyltrimethylammonium bromide (CTAB) were purchased from Fluka A.G. (Switzerland). Ethanol, NaOH, glycine and $Na_2P_2O_7{\cdot}10H_2O$ were purchased from Kemika (Croatia) and acetonitrile was from Fisher Scientific (Great Britain). Glutaraldehyde and 3-aminopropyltrietoxysilane (APTES) were from Sigma (USA). Fresh baker's yeast was purchased from Kvasac d.o.o. (Croatia).

2.2. Methods

2.2.1. Permeabilization procedure

One gram of dry yeast cells was suspended in 10 cm^3 0.2% (w/v) CTAB for 15 min with intermittent shaking [22]. The cells were then separated from the detergent solution by centrifugation at 4500 rpm for 15 min. The yeast cells were then suspended and washed with 75 mmol/dm^3 glycine–pyrophosphate buffer, pH = 9, to remove the remains of the detergent and centrifugation procedure was repeated. Before introducing permeabilized cells in to the microchannel (either during the reaction or immobilization procedure), cells were suspended uniformly in 75 mmol/dm^3 glycine–pyrophosphate buffer, pH = 9, in a final concentration of 10^8 cells/cm^3 (determined by counting the number of the cells in Thoma chamber).

2.2.2. Immobilization procedure

The immobilization process was carried out according to procedure described by Stojkovič and Žnidaršič Plazl [23]. Briefly, glass microchannel was firstly cleaned with 4 mol/dm^3 NaOH, washed with deionized water and treated with 5 mol/dm^3 HNO_3 for 1 h at 90 °C. After washing with deionized water, microchannel surface was silanized with 10% APTES aqueous solution for 24 h. This way, a silane layer with amino groups on the surface was obtained. Microchannels were then washed with deionized water