Chromatographic Removal of Product in-Line with Immobilized Enzyme Bioreactor for the Synthesis of NADH

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Summary: A closed loop system for the synthesis of reduced nicotinamide adenine dinucleotide (NADH) from oxidized NAD^+ is described using isolated and purified Alcohol dehydrogenase (ADH) from Baker's yeast, immobilized on phenolic resin by covalent attachment. The NADH formed is removed by in-line diethylaminoethyl cellulose (DEAE-cellulose) ion exchange column and the progress of the reaction is monitored by high performance liquid chromatography (HPLC). The effect of reagent concentrations and key physical parameters are optimized for the synthesis. Calibration data is linear over the range of 0.2 - 1.0 mM NAD^+ with relative standard deviation (n = 5) in the range 0.6 – 1.9%. The ADH immobilized onto phenolic resin was stable for 3 months at 4°C and lost its activity of 4.0±1.2% at room temperature during synthesis.

Introduction

Nicotinamide adenine dinucleotide (oxidized form NAD^+, reduced form NADH) is an important coenzyme which functions in many dehydrogenase catalyzed reactions, such as those used in the determination of ethanol, lactate, malate, pyruvate and amino acids. The analytical procedures based on such reactions normally involve monitoring the formation or consumption of NADH on the basis of its absorbance at 340 nm or fluorescence at 450 nm; NAD^+ does not give a response at these wavelengths. The regeneration of NAD^+ from NADH is important in the development of sensitive immunoassays and in preparative enzymatic synthesis [1, 2]. Moreover, NADH can be used as medication for Parkinson patient's [3]. Most importantly from biotechnological point of view, NADH is expensive than its oxidized form NAD^+ that makes its production economically viable.

Most industrial enzymatic processes are carried out batch-wise with soluble enzymes, but these processes are very limited, mainly for economic reasons such as low efficiency and high cost [4]. One approach to solve these problems is the use of immobilized enzymes which reduces the cost by reusing the enzyme; the processes can be operated continuously and can be readily controlled; the products can easily be separated and enzyme activity and stability can be altered favorably by immobilization [5, 6]. Bioreactor systems that utilize immobilized enzymes and cells offer many advantages when compared with native enzyme or intact cell systems. Thus, many attempts have been made to design and use new types of bioreactor systems in order to improve bioreactor performance and to enhance productivity [7, 8]. In these bioreactor systems, however, relatively low substrate concentration has been used for the biotransformation reactions when compared with chemical reactions, due to various limitations. A recirculating continuous bioreactor-separator combination system has been reported with its practical applications for high purity product (99.95%, from L-aspartate to L-alanine) [9].

Several methods are available for monitoring pyridine nucleotides (NAD^+ and NADH). These include; an electro-enzymatic method to regenerate NAD^+ by co-immobilizing alcohol dehydrogenase / NADH oxidase coated on electrodes which in-tern produced hydrogen peroxide [10] and an amperometric NADH-sensing electrode which is used for measuring alcohol and NADH, based on substrate recycling with the dehydrogenase catalyzed reaction [11, 12]. However, the immobilization and co-immobilization of enzymes at the surface of electrode suffer from low density, poor reproducibility and short lifetime of analytical sensor [13].

In the present study, we report the isolation and purification of alcohol dehydrogenase (ADH) from Baker's yeast, immobilization on phenolic resin and its use in a closed loop manifold for synthesis of

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NADH from NAD^+. The NADH formed is removed by using an in-line DEAE-cellulose ion exchange resin column and the progress was monitored by HPLC. The reduction of NAD^+ is based on the following reaction catalyzed by ADH:

$$CH_3CH_2OH + NAD^+ \xleftrightarrow{ADH} CH_3CHO + NADH + H^+$$
$$\lambda max = 340 \text{ nm}$$

Results and Discussion

Optimization Studies of Closed Loop System

The experimental conditions for the activity measurement of immobilized alcohol dehydrogenase were optimised by univariate approach. Various parameters were characterized i.e. reagent concentrations, phosphate buffer pH, flow rate and temperature. All these studies were performed with a 1.0 mM NAD^+ standard solution.

The efficiency of immobilized Alcohol dehydrogenase is significantly dependent on reaction conditions. In the proposed closed loop system the effect of phosphate buffer (100 mM) pH in the circulating solution was characterized over the range of 7.5 – 9.0. Maximum conversion of NAD to NADH was observed at pH 8.8 shown in Table-1, above this absorbance was reduced. At pH > 9.0, the destruction of NAD^+ is rapid, whereas NADH is destroyed below pH 6.0 [14], therefore, phosphate buffer (100 mM, pH 8.8) was selected and used for subsequent studies.

The concentration of ethanol plays a vital role in this enzymatic process. The conversion by the enzyme under physiological conditions is unfavorable; therefore, the ethanol quantity must be large enough to change the direction of reaction. Therefore, the influence of ethanol concentration was investigated in the range 0.1 - 3.0% (v/v) using optimized phosphate buffer (100 mM, pH 8.8). As shown in Table-1, the absorbance increased up to 1.5% (v/v) above which a slight decrease in absorbance was observed due to effect on immobilized ADH column. Therefore ethanol solution of 1.5% (v/v) was used subsequently.

Table-1:Effect of Variables on the Conversion of NAD^+ to NADH Using Immobilized Alcohol Dehydrogenase in a Closed Loop System.

pH (Phosphate buffer, 100 mM)	7.5	8.0	8.5	8.8	9.0
Absorbance*	0.10	0.109	0.118	0.170	0.162
Ethanol (%)	0.5	1.0	1.5	2.0	2.5
Absorbance*	0.120	0.160	0.205	0.195	0.180
Flow rate (ml min^{-1})	0.1	0.3	0.5	0.7	1.0
Absorbance*	0.090	0.105	0.090	0.075	0.55
Temp. (°C)	20	30	40	50	60
Absorbance*	0.060	0.075	0.090	0.110	0.120

*Mean of three readings.

The flow rate is an important factor influencing the absorbance signal magnitude during the enzymatic reaction taking place in the circulating solution. Variable flow rate of the circulating solution was calibrated over the range 0.1 – 1.0 ml min^{-1} in terms of NAD^+ to NADH conversion and sample throughput. Maximum absorbance was observed at a flow rate of 0.3 ml min^{-1}, but due to sample dispersion, flow rate of 0.5 ml min^{-1} was used to avoid greater dispersion.

The effect of temperature on the activity of immobilized ADH column was studies over the range 30 – 60°C. There was an increase in absorbance with increase in temperature up to 50°C, above this absorbance reduced. However, the column was maintained at 30°C in closed loop system to protect the enzyme from denaturation and to increase the lifetime of the enzyme reactor.

Biotransformation

Under the optimized conditions, the NAD^+ standards in the range of 0.2 - 1.0 mM, prepared in phosphate buffer (100 mM, pH 8.8), containing 1.5% ethanol, were treated with commercially available

Table-2. Calibration Data for NAD^+.

NAD^+ (mM)	Commercial ADH Absorbance*	RSD (%)	Lab. Isolated ADH Absorbance*	RSD (%)
0.2	0.034	1.4	0.022	1.7
0.4	0.066	1.2	0.042	1.9
0.6	0.096	1.7	0.064	1.6
0.8	0.125	0.6	0.087	1.2
1.0	0.152	1.0	0.110	1.7

*Mean of four readings.

and laboratory isolated immobilized ADH columns. Calibration data obtained is shown in Table-2. The correlation coefficient (r^2) was 0.9984 and 0.9994 (n=5) with regression equations y = 0.1519 x+ 0.003 and y = 0.1096 x – 0.001 [y = absorbance, x = concentration [mM]] respectively. The relative standard deviation was 0.6 – 1.9 % (n = 4) over the range studied.

The stability of circulating solution was also studied composed of phosphate buffer (100 mM, pH 8.8), 2.0 mM NAD^+ and 1.5% ethanol in the reservoir and circulated through immobilized ADH reactor and then kept stoppered at 4°C. The same circulating solution was used for various times at room temperature. No significant change in the absorbance was observed.

The percentage conversion of NAD^+ to NADH was studied by taking 2.0 mM NAD^+ under optimized conditions. The mixture was circulated for 30 min and the percentage conversion was calculated from the absorbance measurement by taking the absorbance value of NADH standard of 2.0 mM as 100%. The maximum conversion 23 ± 1.5% was observed after 30 min, which is due to unfavorable physiological conditions of NADH formation. The degree of conversion in principle can be increased in several manners. i) by increasing pH >10, a 3 fold percent conversion was observed but this pH value can not be used due to the relatively low stability of NAD^+ and enzyme ADH in high alkaline media. ii) the removal of the byproduct acetaldehyde is also a possibility, but that will make the system more tedious and make the purification step more difficult and iii) the best possibility would be the trapping of NADH formed and which has been attempted.

Separation of NADH Formed by Ion-exchange Column Chromatography

It is known that NADH has a higher affinity for weak ion-exchangers than NAD^+ [15]. A. 2.0 g of DEAE-cellulose was packed in a column and experiments were carried out with standard mixtures of NAD^+ and NADH to check the binding capacity of NADH to the resin column. The arrangement is shown in Fig. 1. The 100% absorbance of NADH in the mixture was taken first prior to passing it through resin column. The decrease in absorbance was attributed to that bound to the resin. The results obtained are given in Table-3.

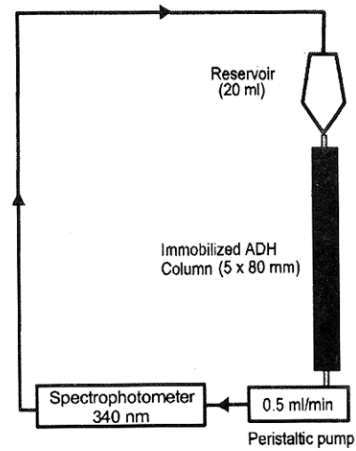

Fig. 1. Experimental set-up for binding NADH to the ion exchange column.

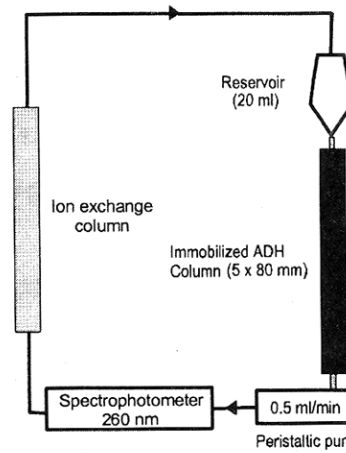

Fig. 2. Experimental set-up for an in-line incorporation of ion exchange column. Reservoir containing 2.0 Mm NAD^+, 1.5% ethanol in phosphate buffer (20 mM, pH 8.8, total volume 20 ml), propelled through both the columns for 60 min. Total nucleotide absorbance at 260 nm was monitored which decreased with time.

Table-3. Conversion of NAD^+ to NADH After Various Time Intervals.

Time (min)	05	10	20	30	60
*NADH bound (%)	22	35	60	81	93
*NADH eluted (%)	10	54	73	80	87

*The values given are the best of four runs.

Binding condition of NADH to the resin: DEAE-Cellulose column equilibrated with phosphate buffer (20 mM, pH 8.8), NADH standard solution (1.0 mM) prepared in the above mentioned buffer was applied to the column and percentage binding of the NADH with time was studied. Flow rate was kept 0.5 ml min^{-1}. The NADH bound to the resin was eluted with phosphate buffer (50 mM, pH 8.8), after removing the previous solution from the column. In case of binding the decrease in absorbance was followed, while in case of elution the increase in absorbance at 340 nm was measured.

Similarly NAD^+ solution were taken and treated in the same way. It was observed that less than 10% of NAD^+ is bound to the ion-exchanger under these conditions. But in case of mixtures an excess of NADH can also displace this small amount of NAD^+ as the later is preferentially bound. The elution of NADH from the column is practically quantitative.

In-Line Incorporation of Ion-exchange Column for Trapping NADH Formed

The schematic diagram of the system is shown in Fig. 2. After circulation through both the columns for 60 min., the ion-exchanger column was removed;

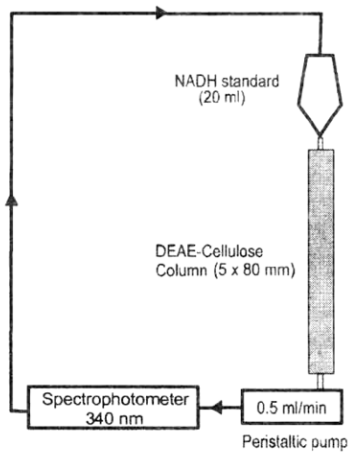

Fig. 3. Gradient HPLC separation of NAD^+ and NADH; flow rate 1.5 ml min^{-1}, column C-18 (3.9 mm x 30 cm, Bondapak), ambient temperature, wavelength 254 nm, variable wavelength uv detector (Model 481, Waters).

the NADH eluted as above and from the absorbance the percentage NADH formed and eluted was calculated. Almost 70% of the NAD^+ initially used was converted into NADH; the conversion being 3 times, compared to the system when ion-exchange column was not applied. For repeating the process, fresh ion-exchange column was used and the utilized column regenerated and equilibrated for fresh application. The NADH preparation is lyophilized and stored as commercial preparation for further usage.

Separation of NAD^+ and NADH by HPLC

A method based on HPLC for the separation of mixture of NAD^+ and NADH was also established to check the purity of the product formed and to monitor the reaction when needed. Fig. 4 shows a typical chromatogram of NAD^+ and NADH, showing the resolution of a mixture of nicotinamide adenine dinucleotide in its reduced and oxidized forms.

Experimental

Materials and Methods

Alcohol dehydrogenase (ADH; Alcohol: NAD^+ Oxidoreductase; EC 1.1.1.1, ex yeast, isolated and purified from active dry yeast; 308 units mg^{-1} according to procedure reported [16], NAD^+ (free from acid, 100%), NADH (disodium salt, 98%), 3-amino-propyltriethoxysilane, glutaraldehyde, and diethylaminoethyl-cellulose (DEAE-cellulose) anion exchange resin (particle size 40 – 120 μ; capacity

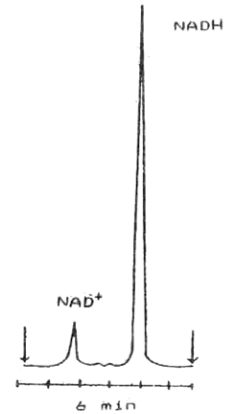

Fig. 4. Closed loop system for the conversion of NAD^+ to NADH.

3.5±0.5 meq/g) were obtained from Sigma (St. Louis, MO. USA). All other reagents were analytical grade (E. Merck, Darmstadt, Germany) and deionsied water was used throughout.

Preparation of Crude Extract

200 g of Baker's yeast powder was homogenized with 500 ml of phosphate buffer (0.05 M, pH 8.5) for 2.5 h at room temperature with constant stirring and the homogenate was centrifuged at 10000 * g for 30 min. The supernatant was brought to 55°C for 15 min, after cooling the mixture was centrifuged and the clear supernatant was stored at 4°C over night.

Enzyme Purification

To the supernatant from yeast's crude extract preparation (100 ml), 50 ml of chilled acetone was added slowly with stirring for about 30 min at –2°C in a dry ice alcohol bath. The resulting precipitate was collected by centrifugation 10000 *g and suspended in 50 ml of cold water, dialyzed against large volumes of phosphate buffer (0.001 M, pH 7.5) for 12 h with several changes of buffer and the insoluble material was removed by centrifugation. To the supernatant, solid $(NH_4)_2SO_4$ was added with constant stirring for 30 min to 36% saturation at 4°C. The precipitate was collected and dissolved in 20 ml of water containing 2.0 g of ammonium sulfate. This solution was subjected to a column (Ag 1-X2, 60 x 3.0 cm) equilibrated with phosphate buffer (0.1 M, pH 8.0) and eluted at a flow rate of 50 ml h^{-1}, 5.0 ml fractions were collected. The unbound material was washed with 500 ml of the same buffer and the enzyme was eluted by a linear gradient (0 – 400 mM) of sodium chloride. The specific activity and percentage yield for each fraction are listed in Table-4.

Enzyme Assay

ADH was assayed by monitoring the oxidation of ethanol coupled with the NAD^+ reduction to NADH. The change in absorbance was monitored at 340 nm. One unit of enzyme is defined as the amount of enzyme, which converts 1.0 µmol of ethanol to acetaldehyde per min. at optimum conditions.

Protein Assay

Protein concentration was determined by Lowry method [17] using bovine serum albumin as a standard.

Table-4:Purification of Alcohol Dehydrogenase from Baker's Yeast.

Fraction	Total units	Total protein	Specific activity	Purifi-cation	Yield
First extract	148000	14800	10	1	100
Acetone ppte.	133000	950	140	14	90
36% $(NH_4)_2SO_4$	74000	264	280	28	50
Column chromat.	66000	214	308	31	45

Phenolic Resin Preparation

11 g of hydroquinone was dissolved in boiling HCl (300 ml, 35%) and aqueous formaldehyde solution (38%, 40 ml) was added dropwise to refluxing hydroquinone solution. Dihydroxydiphenyl methane (DPM) was formed as brown gelatinous suspension, which was refluxed for further 3 h before the addition of cold water. DPM next react slowly with formaldehyde to add more methylol groups. These react quickly with phenol to produce higher molecular weight product. The gel having a settled volume of about 30 ml was dried by lyophilization. The dried mass of brown colored resin (with -OH group on the surface) [18] was used as a support for enzyme immobilization.

Immobilization Procedure

The enzymes were immobilized on phenolic resin by cross-linking with glutaraldehyde according to procedures reported previously [19, 20]. The ADH 5.0 mg (350 Units/mg, commercially available) and 10.0 mg (308 Units/mg, Lab. isolated) dissolved in 5.0 ml of cold phosphate buffer (100 mM, pH 6.0) were separately mixed with 5.0 g aliquots of the derivatized phenolic resins and incubated overnight at 4°C. After the immobilization reaction, the water phase was recovered and the resin was washed with a small portion of phosphate buffer (100 mM, pH 7). The protein content of the residue was measured according to the procedure reported [12] to evaluate the yield of the immobilization. Almost 90% and 70% of the enzymes were bound to the supports respectively. The immobilized enzymes were packed in glass columns (80 x 5.0 mm), washed with phosphate buffer (100 mM, pH 8.5) and utilized as needed. The immobilized enzymes were utilized for about 300 h without any appreciable change in their activity. The enzymatic activity was completely preserved after three months storage at 4°C in phosphate buffer (100 mM, pH 8.8).

Closed Loop System

The closed loop system containing the immobilized enzyme column is shown in Fig. 4. The reservoir contained 20 ml of solution for circulation. The solution was 1.0 mM NAD^+ in phosphate buffer (100 mM, pH 8.8) containing 1.5% ethanol. The system was composed of a peristaltic pump (LKB, Broma, Finland) connected with the reservoir and the alcohol dehydrogenase column. The flow rate was maintained at 0.5 ml min^{-1}. The circulating time was 20 min. The enzymatically produced NADH was monitored at 340 nm using a spectrophotometer (LKB, Model 4040, Finland) with a flow through cell (30 µl). All experiments were carried out at room temperature.

Assay of NADH Production by HPLC

Analytical separation was performed using the liquid chromatography (Waters, Model 510 HPLC system) with a bondapak C-18 column (3.9 mm x 30 cm). The column was loaded with 10 µl incubation mixture; the flow rate was 1.5 ml min^{-1} and the detection was performed at 254 nm with a variable wavelength UV detector (Model 481). Elution was performed with 100 mM KH_2PO_4/CH_3OH gradient. Prior to the analysis, the column was equilibrated with mobile phase for 1 h at a flow rate of 2.0 ml min^{-1}.

Conclusions

ADH from Baker's yeast was isolated, purified and immobilized on phenolic resin. Several materials including cellulose, silica gel, controlled pore glass, alkylamine glass and nylon membrane available commercially have been used as supports for immobilization of enzymes which makes the process expensive on commercial scale. The phenolic resin prepared in the laboratory has proved to be excellent in terms of its low cost, stability, giving an active product, which does not swell on packing having good flow properties. The cross linking method used for phenolic resin has proved to be extremely good and gave good product which was stable and no appreciable change in activity was recorded in daily usage. The activity of commercially available ADH compared with laboratory isolated and purified showed no significant difference in terms of limit of detection and range. The introduction of ion exchange resin column in-line has proved to be a novel idea and can be applied to a number of other cases as well.

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