# APPLICATION OF NADH OXIDASE IN FIBRE OPTIC BIOSENSORS

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**Summary**

A new fully reversible fibre-optic detection system based on the detection of NADH-fluorescence is presented. NADH oxidase (EC: 1.6.99.3) was used to regenerate NADH that is needed for the oxidizing reaction of alcohols and aldehyds by different dehydrogenases. In the oxidation reaction $NAD^+$ was reduced to NADH and the increase of fluorescence was monitored by a fibre-optic detection system. The NADH-fluorescence decreased in the absence of substrate due to the oxidation of NADH by NADH oxidase.
Different types of NADH oxidase (*Thermus thermophilus*, *Thermus aquaticus* und *Bacillus licheniformis*) were studied in respect to their application in optical sensors. Only NADH oxidase of *B. licheniformis* proved to be active and stable at any assay conditions even in the absence of FAD.

## Introduction

The oxidation of NAD(P)H to $NAD(P)^+$ is of great importance in analytical biochemistry due to regeneration and quantitative determination of coenzymes. The detection of NADH or NADPH plays a central role in clinical and food technological analysis, because these cofactors are used by dehydrogenases (EC 1.1.1). More than 250 dehydrogenases are available for the determination of various analytes /1/.
The use of NADH oxidase (EC 1.6.99.3) offers the possibility to create a fully reversible fibre optic detection system for substrates of dehydrogenases based on the fluorimetric detection of NADH. In a dehydrogenase reaction (eq. 1) $NAD^+$ is reduced to NADH, The increase of fluorecence can be monitored by a fibre optic detection system. In the absence of substrate the NADH fluorescence decreases due to the oxidation of NADH by NADH oxidase (eq.2).

Dehydrogenase reaction:
substrate (reduced) + $NAD^+$ + $H_2O$ --> product (oxidized) + NADH + $H^+$ (eq. 1)

Oxidase reaction:
NADH + $H^+$ +$O_2$ --> $NAD^+$ + $H_2O_2$ (eq. 2).

## Experimental

Different types of NADH oxidase (*Thermus thermophilus* /2/, *Thermus aquaticus* /3/ and *Bacillus licheniformis* /4/) were studied as shown in Fig. 1, in respect to their application in optical biosensors.

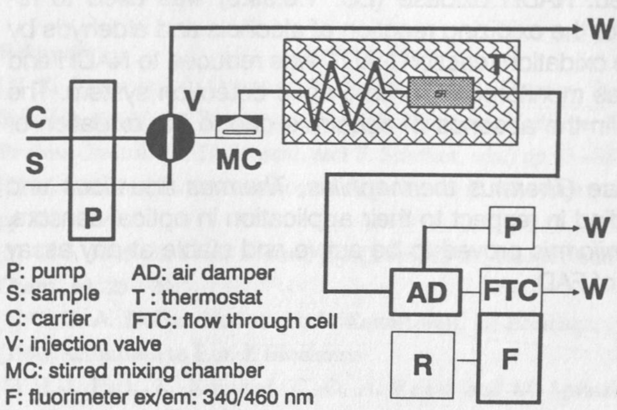


**Fig.1**
*FIA arrangement to perform the fluorometric determination of apparent enzyme activities of immobilized NADH oxidases. NADH samples S were injected by an injection valve V (100 µl) into a carrier stream C (50 mM potassium phosphate buffer). The injected sample pumped with a peristaltic pump P was diluted and buffered with a stirred minxing chamber MC (200 µl), thermostated in a water bath T, and converted in an enzyme reactor ER containing immobilized NADH oxidase. An air damper AD protected the flow through cell FTC of the fluorometer F from air bubbles. The detected fluorescence (ex/em: 340/460 nm) was recorded on a recorder R. All solutions were collected in a waste container W. The decrease of NADH fluorescence due to the activity of immobilized NADH oxidase was corrected by a dummy reactor for intrinsic fluorescence changes, e.g. changes by varying temperatures or pH.*

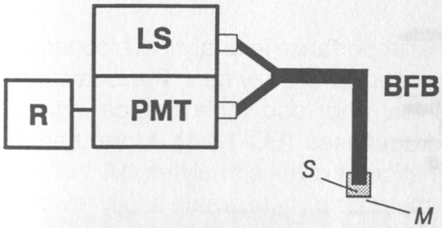


**Fig. 2**
*Schematic diagram of the optical fibre sensor for the determination of volatile substrates based on dissolved dehydrogenases, NADH oxidase and NAD$^+$. Light was guided from a light source LS through a bifurcated fibre bundle BFB to the sensor tip S. The dissolved enzymes and coenzymes were separated by a gas permeable membrane M from the sample solution. The fluorescence light is guided back to the photomultiplier PMT, and the signal is printed at the recorder R.*

A schematic diagram of the optical fibre sensor for the determination of volatile substrates based on dissolved dehydrogenases, NADH oxidase and NAD$^+$is shown in Fig. 2.

Results & discussion

Enzyme activities of the immobilized NADH oxidases were fluorimetrically determined in an FIA system under various conditions. Immobilization resulted in a broader temperature and pH profile of enzyme activity compared to the soluble enzymes. Especially the activity of the immobilized NADH oxidase from *B. licheniformis* is nearly temperature independent in a range from 20 to 55°C. The immobilized NADH oxidase from T. thermophilus shows a temperature optimum at 65°C, at 20°C only 40 % of enzyme activity remain. At temperatures that are normally used for biosensor applications (20 to 30°C) the NADH oxidase from the mesophilic *Bacillus* offers the highest activity. The activities of the NADH oxidases depend on the different concentrations of FAD as shown in Fig. 3.

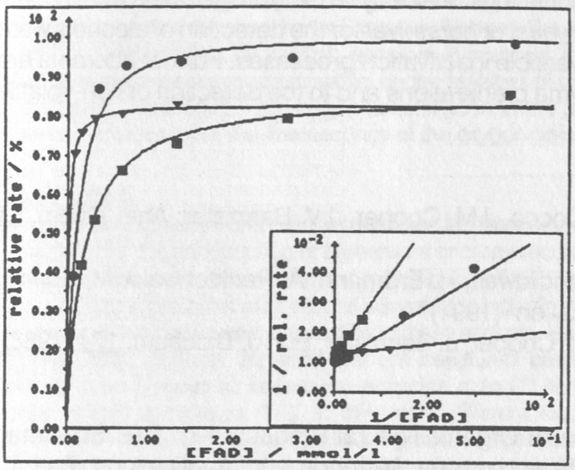


**Fig.3**
*Michaelis-Menten plots of the activities of NADH-oxidases from T. thermophilus (circle), T. aquaticus (triangle) and B. licheniformis (square). The conditions of enzyme reactions are given in Tab.1. Corresponding Lineweaver-Burk plots are shown in the small incorporated figure.*

Tab.1
Apparent $K_M$-values of FAD at pH 7.2 of immobilized NADH-oxidases from *Thermus thermophilus, Thermus aquaticus* and *Bacillus licheniformis*

| Immob. NADH-Oxidase from | conditions | KM-values [mol/l] |
|---|---|---|
| *T. thermophilus* | *60°C, injection of 0.2 mM NADH* | *9.98 E-6* |
| *T. aquaticus* | *60°C, injection of 0.2 mM NADH* | *1.50 E-6* |
| *B. licheniformis* | *60°C, injection of 0.4 mM NADH* | *1.95 E-6* |

The apparent KM-values of FAD of the given immobilized enzymes are given in Tab.1.

In contrast to the NADH oxidases from the *Thermus* strains the enzyme from *Bacillus* is active even in the absence of FAD. This is a requirement for enzymes being applied in optical biosensors based on the detection of NADH fluorescence intensity, because of the high self-fluorescence of FAD/5/. These results indicate that the NADH oxidase from *Bacillus* is the most suitable one for applications in optical sensors.

Combinations of different types of dehydrogenases with the NADH oxidase from *B. licheniformis* were tested for the determination of volatile substrates, e.g. alcohols or aldehydes. In case of aldehyde dehydrogenase (EC 1.2.1.5) from *yeast* the response towards 10 mM butyraldehyde solutions was reversible within 6 min using a hydrophobic polypropylen membrane. Combinations of aldehyde dehydrogenases with alcohol deyhdrogenases (EC 1.1.1.1) from *yeast* or *horse liver* for the detection of alcohols were instable due to impurities and irreversible inactivation processes. Further attempts are directed towards more stable enzyme preparations and to the detection of non-volatile substrates.

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