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ORIGINAL RESEARCH PAPER

A high effective NADH-ferricyanide dehydrogenase coupled with laccase for NAD^+ regeneration

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Abstract
Objectives To find an efficient and cheap system for NAD^+ regeneration
Results A NADH-ferricyanide dehydrogenase was obtained from an isolate of *Escherichia coli*. Optimal activity of the NADH dehydrogenase was at 45 °C and pH 7.5, with a K_m value for NADH of 10 μM. By combining the NADH dehydrogenase, potassium ferricyanide and laccase, a bi-enzyme system for NAD^+ regeneration was established. The system is attractive in that the O_2 consumed by laccase is from air and the sole byproduct of the reaction is water. During the reaction process, 10 mM NAD^+ was transformed from NADH in less than 2 h under the condition of 0.5 U NADH dehydrogenase, 0.5 U laccase, 0.1 mM potassium ferricyanide at pH 5.6, 30 °C
Conclusion The bi-enzyme system employed the NADH-ferricyanide dehydrogenase and laccase as catalysts, and potassium ferricyanide as redox mediator, is a promising alternative for NAD^+ regeneration.

Keywords Coenzyme regeneration · Coupled enzymes · Laccase · NAD^+ · NADH dehydrogenase · Potassium ferricyanide

Introduction

NAD^+ plays a major role in many biological oxidation–reduction reactions and its associated oxidoreductase has potential applications in synthetic chemistry (Chenault and Whitesides 1987). However, one fundamental obstacle to the large-scale use of NAD^+-dependent oxidoreductases is the high cost of the cofactor when used as a stoichiometric reagent. Therefore, for the economic reasons, a favorable thermodynamic route for regeneration of NAD^+ is desirable. Significant effort has been made to overcome the limitation of nicotinamide cofactors for possible large-scale industrial applications. Well-established enzymatic methods for NADH regeneration consist of formate dehydrogenase (Bommarius et al. 1998), alcohol dehydrogenase (Grunwald et al.

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1986), and glucose dehydrogenase (Kaswurm et al. 2013) etc. There are, however, different strategies for regenerating NAD^+, some of which involve enzymatic, chemical, photoelectrochemical, or electrochemical methods. Among these, enzymatic methods seem the most promising approach due to convenience and high efficiency; examples include glutamate dehydrogenase with 2-ketoglutarate (Lee and Whitesides 1985), alcohol dehydrogenase with acetaldehyde (Šalić et al. 2013), and lactate dehydrogenase with pyruvate (Chenault and Whitesides 1989). NADH oxidases are also used to catalyze NADH oxidation to NAD^+ with O_2 reduced to H_2O_2 (Geueke et al. 2003; Riebel et al. 2003). Moreover, two-enzyme systems are used to catalyze NAD^+ regeneration (Aksu et al. 2009). However, many enzymatic methods used for the NAD^+ regeneration needed the addition of co-substrate or catalase (Itoh et al. 1992).

In this contribution, we present a new alternative to regenerate NAD^+ efficiently using NADH dehydrogenase obtained from *E. coli*, coupled with laccase from *Agaricus bisporus*. In the reaction system, potassium ferricyanide served as hydrogen acceptor from NADH (Kulikova 2005), and laccase was employed for the regeneration of electron acceptor potassium ferricyanide (Wang et al. 2013). Firstly, NAD^+ was regenerated by NADH dehydrogenase and potassium ferricyanide was concomitantly reduced to potassium ferrocyanide. Secondly, potassium ferrocyanide was continuously oxidized to potassium ferricyanide by laccase, at the same time O_2 was converted to H_2O (Fig. 1). In the whole reaction process, there was no requirement for the addition of a sacrificial substrate or catalase (Jouanneau et al. 2006); only H_2O was generated as by-product. Taking these advantages into account, the approach is attractive not only from the economic point of view, but also from ecological considerations.

Materials and methods

Chemicals

All chemicals used were the highest grade and were purchased from Sinopharm Chemical Reagent Corporation (Nanjing, China). Toyopearl DEAE-650M was obtained from Tosoh Corporation (Osaka, Japan).

Organism and growth

The bacterial strain used to produce NADH dehydrogenase was isolated from soil at Nanjing University of Science and Technology (Nanjing, China). It was identified by 16S rRNA sequencing as *Escherichia coli* with the designation of JZW506. It was grown in lysogeny broth (100 ml) in a 500 ml Erlenmeyer flask at 37 °C, 120 rpm for 18 h. The culture was centrifuged at 5000×*g* for 10 min and the cells were washed with distilled water three times.

Purification of NADH dehydrogenase

All procedures were performed under 4 °C or in an ice-water bath. Washed cells (from 1 l culture) were resuspended in 10 ml distilled water containing 200 µl mercaptoethanol (0.5 %, v/v), 1 ml Triton X-100 (1 %, v/v), and then were disrupted ultrasonically over 20 min. Ammonium sulphate fractionation was carried out collecting the pellet that formed between 40 and 70 % saturation. This was dissolved in 10 mM sodium phosphate buffer (pH 8). After being dialyzed

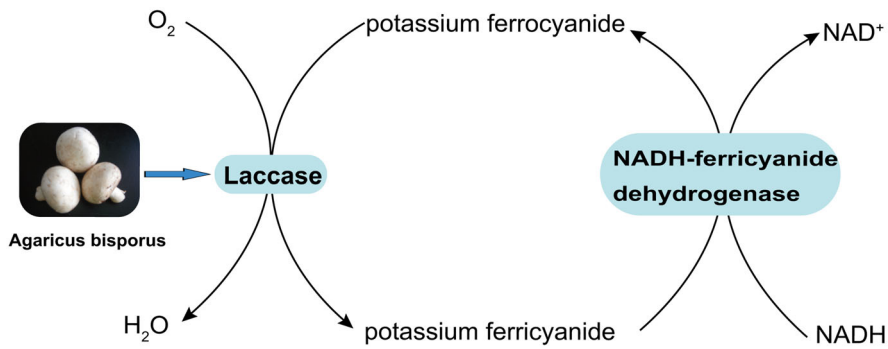

Fig. 1 Bi-enzyme system of NADH dehydrogenase and laccase for NAD^+ regeneration

against the same buffer, the crude preparation was applied to Toyopearl DEAE-650 M column (1.5 cm × 20 cm) which had been equilibrated with 10 mM sodium phosphate buffer (pH 8). The enzyme was eluted with a linear 0–1 M NaCl gradient in 10 mM sodium phosphate buffer (pH 8, 100 ml) at a flow rate of 1.0 ml/min and the active fractions were collected.

Biochemical properties of NADH dehydrogenase

The optimal pH of NADH dehydrogenase was analyzed at 30 °C in following buffers (final concentration, 50 mM): citrate/phosphate buffer (pH 5.6-8), and Tris/HCl buffer (pH 8–9). The optimal temperature of NADH dehydrogenase was analyzed at various temperatures from 25 to 70 °C in Tris/HCl buffer (pH 7.5, 50 mM). The pH stability of the NADH dehydrogenase was analyzed by incubating the enzyme at different pH buffers (pH 6–9, 50 mM) at 45 °C for 6 h. The thermal stability of the NADH dehydrogenase was analyzed by incubating the enzyme at different temperatures (30–50 °C) for 6 h. The Michaelis constant (K_m) of NADH dehydrogenase toward NADH was determined at the condition of 0.2 mM potassium ferricyanide, Tris/HCl buffer (pH 7.5, 50 mM) and 45 °C using Lineweaver–Burk method. The K_m value of NADH dehydrogenase toward potassium ferricyanide was determined at the condition of 0.1 mM NADH, citrate/phosphate buffer (pH 5.6, 50 mM) and 30 °C.

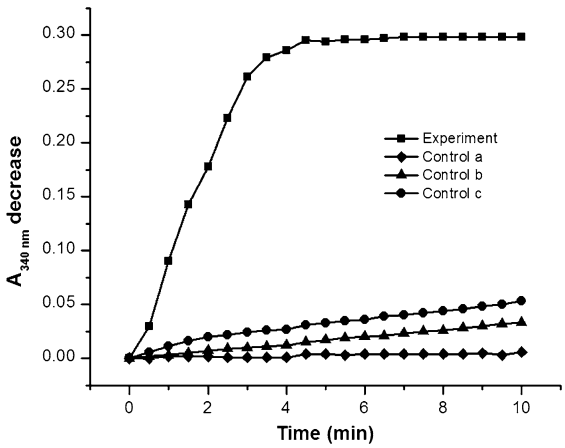

Fig. 2 The test of NADH dehydrogenase activity toward NADH. (*Filled square*) experiment: 1 mM potassium ferricyanide, 100 μM NADH, and a suitable amount of enzyme in citrate/phosphate buffer (pH 5.6, 50 mM) at 30 °C (*filled diamond*) control a: 100 μM NADH in citrate/phosphate buffer (pH 5.6, 50 mM) at 30 °C; (*filled triangle*) control b: 100 μM NADH, and a suitable amount of enzyme in citrate/phosphate buffer (pH 5.6, 50 mM) at 30 °C; (*filled circle*) control c: 1 mM potassium ferricyanide, 100 μM NADH in citrate/phosphate buffer (pH 5.6, 50 mM) at 30 °C

Enzyme activity assays

The NADH dehydrogenase activity was assayed at 30 °C by monitoring the decrease in NADH absorbance at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$) for 1 min in citrate/phosphate buffer (pH 5.6, 50 mM) containing 1 mM potassium ferricyanide, 100 μM NADH, and a suitable amount of enzyme. One unit of enzyme activity was defined as the amount of enzyme which decreased 1 μmol NADH per min.

Laccase

Laccase was extracted from fruiting bodies of *Agaricus bisporus.* The experiments of laccase purification were performed as described by Wang et al. (2013).

Laccase activity was determined at 30 °C by following the increase in the absorbance of potassium ferricyanide at 420 nm ($\varepsilon_{420} = 1.04\ mM^{-1}\ cm^{-1}$) (Wang et al. 2013). The assay contained 1 mM potassium ferrocyanide, a suitable amount of enzyme, and citrate/phosphate buffer (pH 5.6, 50 mM). One unit of laccase activity was defined as the amount of enzyme oxidizing 1 μmol potassium ferrocyanide per min.

The bi-enzyme system for NAD^+ regeneration

To investigate the influence of pH (3–8) on the bi-enzyme system for NAD^+ regeneration, the reaction was carried out by monitoring the decrease of NADH absorbance at 340 nm in 50 mM citrate/phosphate buffer containing 300 μM potassium ferricyanide, 100 μM NADH, 0.05 U laccase, and 0.05 U NADH dehydrogenase. Absorbencies were converted into concentrations of NADH using the molar extinction coefficient ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$). A control experiment was performed with equivalent volumes of distilled water instead of enzyme. To investigate the influence of temperature (30–50 °C) on the bi-enzyme system for NAD^+ regeneration, the reaction was carried out in citrate/phosphate buffer (pH 5.6,

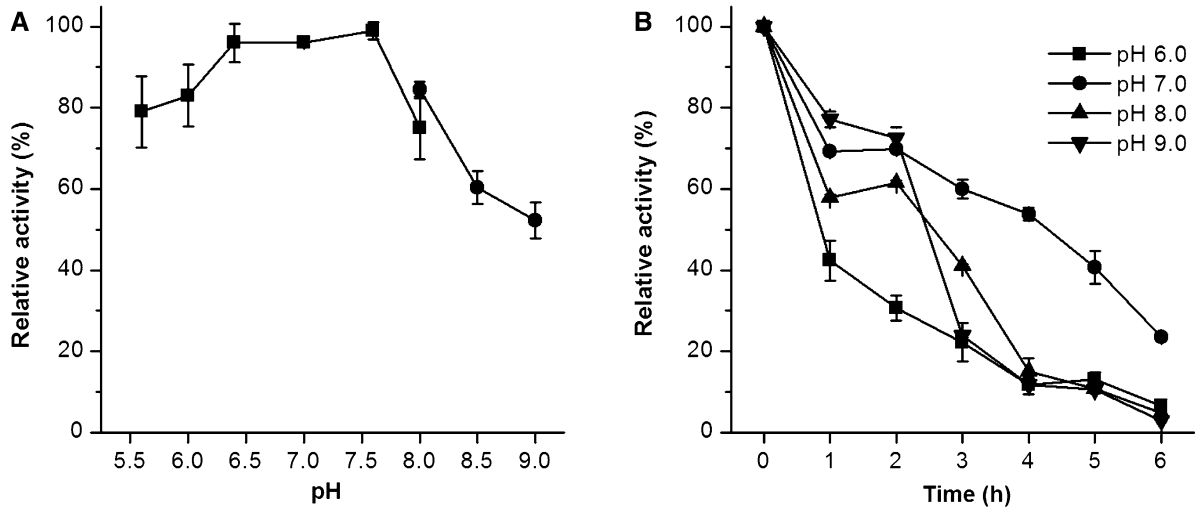

Fig. 3 Effect of pH on activity (**a**) and stability (**b**) of NADH dehydrogenase

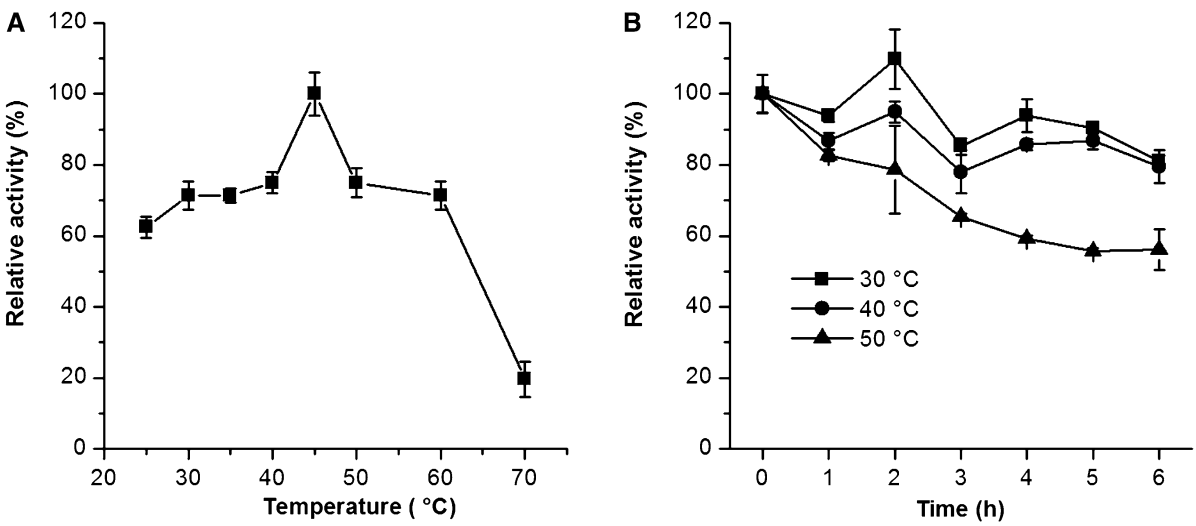

Fig. 4 Effect of temperature on activity (**a**) and stability (**b**) of NADH dehydrogenase

50 mM) containing 100 μM potassium ferricyanide, 1 mM NADH, 0.05 U laccase, and 0.05 U NADH dehydrogenase (Fig. 1). After 30 min, samples were taken and analyzed by HPLC.

To evaluate the efficiency of the chosen conditions, the reaction was performed on a rotary shaking incubator (140 rpm) at 30 °C in citrate/phosphate buffer (pH 5.6, 50 mM) containing 0.1 mM potassium ferricyanide, 10 mM NADH, 0.5 U laccase, and 0.5 U NADH dehydrogenase. A control experiment was performed with 10 mM NADH in citrate/phosphate buffer (pH 5.6, 50 mM). Samples were taken periodically and analyzed by HPLC.

HPLC analysis

The concentrations of NADH and NAD^+ in reaction samples were monitored by HPLC using a C_{18} column (150 mm × 4.6 mm) with methanol/phosphate buffer (6.6 g Na_2HPO_4 l^{-1}, 6.8 g KH_2PO_4 l^{-1}) (6:94, v/v) was used as eluent at 0.6 ml/min at 25 °C. Detection was at 260 nm. A calibration curve was carried out

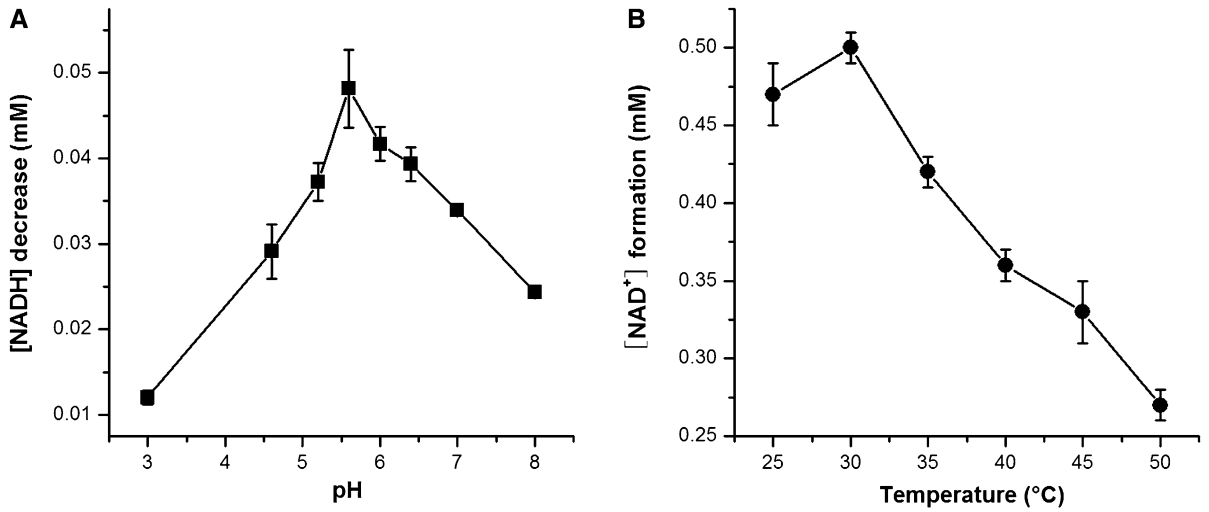

Fig. 5 Effect of pH (**a**) and temperature (**b**) on NADH dehydrogenase and laccase bi-enzyme system for NAD^+ regeneration

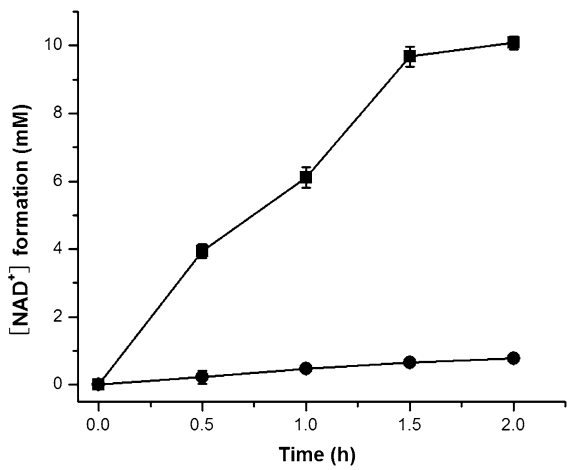

Fig. 6 The regeneration of NAD^+ from NADH by NADH dehydrogenase and laccase bi-enzyme system

with authentic samples of NADH and NAD^+ from 0.01 to 10 mM.

Results and discussion

The properties of NADH dehydrogenase

The 16S rRNA sequence of the isolated strain of *E. coli* has been deposited in NCBI GenBank with accession number KT716302. The bacterium was grown in medium using 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate that emitted fluorescence when exposed to light at 365 nm (data not shown).

After two purification steps (ammonium sulfate fractionation followed by Toyopearl DEAE-650M column), the obtained NADH dehydrogenase was treated as a purified enzyme. Its activity was followed at 340 nm (Fig. 2). Without ferricyanide in the control experiment, no obvious absorption decrease was detected. Figure 2 also shows that low concentrations of NADH were decreased even though potassium ferricyanide was present.

NADH dehydrogenase was optimally active at pH 7.5 and 45 °C (Fig. 3). More than 50 % of the activity was retained after 4 h at pH 7 and at 45 °C. It was not stable at pH 6 or 9, with only about 10 % activity maintaining after 4 h incubation at 45 °C. NADH dehydrogenase was stable under 40 °C, maintaining about 80 % of its activity following incubation for 6 h (Figs. 3, 4). NADH dehydrogenase had K_m of 10 μM for NADH (Supplementary Fig. 1). NADH dehydrogenase had K_m of 37 μM for potassium ferricyanide (Supplementary Fig. 2). Optimal activity of laccase was at 30 °C and pH 3 (Wang et al. 2013).

Regeneration of NAD^+ by NADH dehydrogenase and laccase bi-enzyme system

There is a difference in optimal pH and temperature between NADH dehydrogenase (pH 7.5, 45 °C) and laccase (pH 3, 30 °C). More units of laccase were needed when NADH dehydrogenase was assayed at pH 7.5 due to the activity of laccase at pH 7.5 being very low, and vice versa. Therefore, we investigated

the influence of pH and temperature on the catalysis of the bi-enzyme system, and optimal condition was at pH 5.6 and 30 °C (Fig. 5). We further investigated the stability of NADH dehydrogenase and laccase following incubation at 30 °C in citrate/phosphate buffer (pH 5.6, 50 mM). NADH dehydrogenase and laccase were stable under the optimal condition, maintaining about 50 % and 100 % of their activity after 8 h of incubation (Supplementary Fig. 3).

10 mM NADH was completely converted to NAD^+ in less than 2 h under the chosen condition (Fig. 6). The result clarifies that NADH dehydrogenase can efficiently convert NADH to NAD^+. Further investigations will focus on coupling the NAD^+-regenerating system with NAD^+-dependent oxidoreductases. During the process, we can calculate the turn number of NAD^+ to evaluate the efficiency of the system. It should be mentioned here that potassium ferrocyanide is considered as harmful to the environment and to water organisms. Therefore, studies on replacing potassium ferricyanide by other mediators that are environment ally benign are underway.

Conclusion

We present a cheap, efficient system for NAD^+ regeneration by coupling with NADH dehydrogenase from *E.coli* JZW506 and laccase from *Agaricus bisporus*. The research regarding NAD^+ regeneration suggests that the system could be a potential approach for use in industrial biocatalytic application.

Supplementary information Supplementary Fig. 1—Line weaver-Burk plot of the effect of NADH concentration on NADH dehydrogenase activity. Enzyme kinetic parameters were obtained by measuring the rate of A_{340} value decrease at various concentrations of NADH ranging from 12.5 μM to 400 μM at 45 °C in Tris/HCl buffer (pH 7.5, 50 mM).

Supplementary Fig. 2—Lineweaver-Burk plot of the effect of potassium ferricyanide concentration on NADH dehydrogenase activity. Enzyme kinetic parameters were obtained by measuring the rate of A_{340} value decrease at various concentrations of potassium ferricyanide ranging from 6.25 μM to 200 μM at 30 °C in citrate/phosphate buffer (pH 5.6, 50 mM).

Supplementary Fig. 3—Residual activity of NADH dehydrogenase and laccase following incubation at 30 °C in citrate/phosphate buffer (pH 5.6, 50 mM).

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