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Selection and Microencapsulation of an "NADH-Oxidizing" Bacterium and Its Use for NAD Regeneration

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ABSTRACT

An alternative approach to the regeneration of coenzymes is described here using immobilized microorganisms possessing "NADH-oxidase" function. Bacteria containing NADH-oxidase activity are immobilized by microencapsulation within artificial cells. In this form, the microencapsulated bacteria can recycle NADH back to NAD in the presence of molecular oxygen as an electron acceptor. The only byproduct of the recycling reaction is water. In order to perform the biological regeneration of NAD, the activity of NADH-oxidase was investigated in 13 strains of aerobic bacteria and yeast. The NADH-oxidizing bacteria *Leuconostoc mesenteroides* exhibited the highest activity among the microorganisms tested. The permeabilized bacteria showed 10% of their initial activity after microencapsulation. Light and electron microscopy studies of bacteria loaded microcapsules have been done. Enzymatic properties of microcapsule-immobilized bacteria were investigated in comparison with those of the free enzyme complex. *Leuconostoc mesenteroides,* containing NADH-oxidase, has been microencapsulated together with 3α-hydroxysteroid dehydrogenase (3α-HSDH) for stereospecific steroid oxidation.

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In a batch reactor, 2 mg of NAD, with recycling, allowed the same substrate consumption as 4.4 mg of NAD without recycling. The microencapsulated system can be used repeatedly. The system is functional for 10 h, during which time each molecule of NAD has been used 7.6 times.

Index Entries: NAD^+ recycling, and microencapsulation; hydroxysteroid dehydrogenase, microencapsulation of; microencapsulated bacteria; "NADH-oxidase"; immobilization, of an NADH-oxidizing bacterium; immobilized multienzyme, microencapsulated; enzyme, microencapsulation and regeneration of; biotechnology, and NAD regeneration; artificial cells, and NAD regeneration.

INTRODUCTION

So far in the biotechnology of immobilized multienzyme systems, the problem of cofactor regeneration, requiring its coretention in open reactors, has not found any satisfactory answer. It is obvious that any saving introduced by the reusable nature of immobilized enzymes will be lessened if the system in question has to be continuously supplied with expensive cofactors. One approach is the use of microencapsulation to immobilize both the multienzyme systems and the required cofactors (*1*).

Steroid-dehydrogenases are of considerable interest for stereospecific steroid transformations. These dehydrogenases require freely dissociable electron acceptors such as NAD^+ or $NADP^+$ to achieve catalytic oxidoreductions. Coenzyme regeneration can be accomplished by several alternative methods: (1) enzymatic (*2*); (2) chemical (*3*); (3) electrochemical (*4*). Such systems may pose significant problems: separation of the product and substrate used for regeneration from the reaction product, cost of the substrate used for regeneration, imput of electrical and chemical energy and incompatibility of the process with regeneration conditions. Some of these problems can be avoided by the use of molecular oxygen as the final electron acceptor. NADH-oxidase can regenerate NAD from NADH using only O_2, and producing water (*5,6*). The immobilization techniques previously used (*5,6*) did not allow the retention of the NAD, which has to shuttle between the dehydrogenase and the NADH-oxidase function.

In order to obtain a more active and stable NADH-oxidase, 13 strains of aerobic microorganisms were investigated for NADH-oxidase activity. This report describes the immobilization of whole "NADH-oxidizing" bacteria and their possible use in regenerating NADH when coimmobilized with a dehydrogenase. The immobilization was carried out by the technique of microencapsulation (*7–10*).

MATERIAL AND METHODS

Culture Conditions and Preparation of the NADH-Oxidizing Bacteria

Microorganisms are cultivated aerobically in 500 mL flasks containing 50 mL of an appropriate culture medium at 30°C for 20–30 h on a rotary shaker. Two cultures of cells are harvested, one during the logarithmic phase and the other at the stationary phase of growth. Centrifugation at 10,000*g* for 15 min is followed by washing twice with 0.02*M* phosphate buffer (pH 7.4). The washed cells are resuspended in the phosphate buffer and prepared for the screening test in order to make the "NADH-oxidase" function accessible. Twelve different assays are performed on each organism. Each of the two harvested stages are further divided into two parts: one frozen, the other stored at 4°C. Subsequently each of these samples is tested following two treatments: permeabilization with toluene (10 μL/mL) and sonication. In order to obtain cells on a large scale, *Leuconostoc mesenteroides* is grown to the logarithmic phase at 30°C in 40 L of culture medium in a 60 L fermentor. The culture is aerated at a rate of 30 L/min and agitated at 500 rpm.

Preparation of Semipermeable Collodion Microcapsules

Semipermeable microcapsules are prepared by the method using collodion membranes (*7,8*). The updated microencapsulation procedure, which generates microcapsules with a mean diameter of 80–100 μm, is employed (*9,10*).

Measurement of "NADH-Oxidase" Activity

The enzyme activities of the bacteria (free or microencapsulated) are determined by incubating each of the preparations with 30 mL of sodium pyrophosphate buffer (0.1*M*, pH 8.5), containing NADH ($1.5 \times 10^{-4}M$). Oxygen is provided by bubbling air into the reaction vessel. The reactions are carried out in a rotary shaker (Environ Shaker 18 Lab Line Instruments) at 120 rpm and 30°C. Aliquots are taken at timed intervals and the absorbance read at 340 nm (peak for NADH) on a Cary Model 219 spectrophotometer. Each aliquot is then returned to the reaction vessel so as to keep the reaction volume constant. The amount of NADH-oxidase activity is proportional to the observed decrease in optical density at 340 nm.

Measurement of NAD Recycling

When coupling the NADH-oxidase function with 3α-hydroxysteroid dehydrogenase (3α-HSDH) the disappearance of substrate (androsterone) is followed using an enzyme assay procedure. Since the recycling

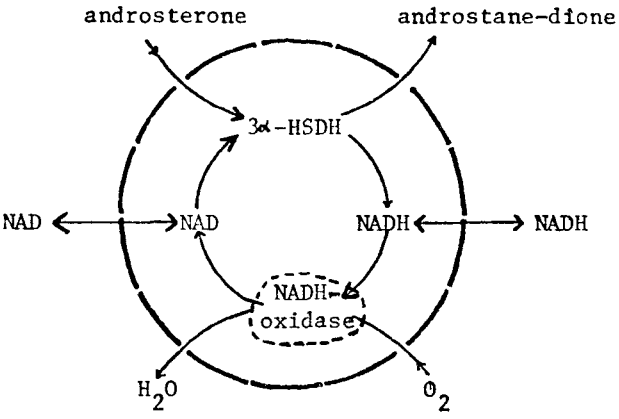

Fig. 1. Schematic representation of the system used for NAD recycling.

system is supplied with androsterone, NAD and O_2, 3α-HSDH becomes the first enzyme in this cycle, shown in Fig. 1.

RESULTS AND DISCUSSION

Screening for NADH-Oxidizing Bacteria

The results of the screening test are summarized in Table 1. The bacteria *Leuconostoc mesenteroides* exhibited the highest specific activity among the thirteen microorganisms tested. It is also evident that bacteria harvested at the end of the exponential growth phase have the highest activity.

Activity of Microencapsulated "NADH-Oxidizing" Bacteria

Two samples of bacteria harvested during late exponential growth were prepared: pure and permeabilized with toluene. A 0.5 mL aliquot of each sample was mixed with 2 mL of Hb (12.5 g/dL), microencapsulated and then assayed. Figure 2 shows some photographs of these microcapsules using both light and transmission electron microscopy (TEM). When using TEM, one can actually identify bacteria inside the cells. Figure 3 illustrates the consumption of NADH by the different bacteria preparations. Pure bacteria that have no activity when in free suspension, exhibit a weak activity after microencapsulation. This is most likely due to some bacteria permeabilized during the microencapsulation process. Controls were done to ensure that no decrease of OD occurred in the presence of control microcapsules. Permeabilized bacteria, microencapsulated after toluene treatment, give better activity (Fig. 3). These results show that bacteria should be permeabilized by treatment with toluene before they are microencapsulated.

TABLE 1
NADH-Oxidase Activity of Various Bacteria and Yeast Tested Following Different Methods of Preparation[a]

Microorganism	Fresh, stationary phase			Frozen, stationary phase			Fresh, logarithmic phase			Frozen, logarithmic phase		
	Pure	Permeabilized	Sonicated	Pure	Permeabilized	Sonicated	Pure	Permeabilized	Sonicated	Pure	Permeabilized	Sonicated
Acetobacter suboxydans	0	0.10	0	0	0.02	0	0	0.55	0.50	0	0.30	0
Azotobacter chroococcum	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus cereus							0.06	0	0	0.10	0.05	0.004
Bacillus coagulans Medium A	0	0.17	0.10	0.06	0.16	0	0	0	0	0	0.18	0
Bacillus coagulans Medium IPP	0	0.08	0.02	0.02	0.22	0.04	0.26	0.32	0	0.22	0.20	0
Bacillus stearothermophilus	0	0	0	0	0.04	0	0	≃0	0	0	≃0	0
Bacillus subtilis							0.16	0.26	0	0.16	0.01	0
Candida tropicalis	0	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli	0	0.39	0	0.07	0.25	0	0	0.34	0	0	0.26	0.14
Leuconostoc mesenteroides A	0	0.18	0	0	0.14	0	0	1.25	0.50	0	0.40	0
Leuconostoc mesenteroides IPP	0	0.23	0	0	0.09	0	0	0.60	0.07	0	1.10	0
Mycobacterium phlei	0	0.03	0.02	0.37	0.13	0.07	0	0.05	0	0.04	0.006	0
Mycoplasma laidlawii												
Mycoplasma mycoides												
Streptococcus fecalis	0	0.02	0	0	≃0	≃0	0	0.02	0	0	≃0	0

[a]Medium A: Kawai et al. (1971).
Medium IPP (Institut Pasteur Production): modified MRS medium.

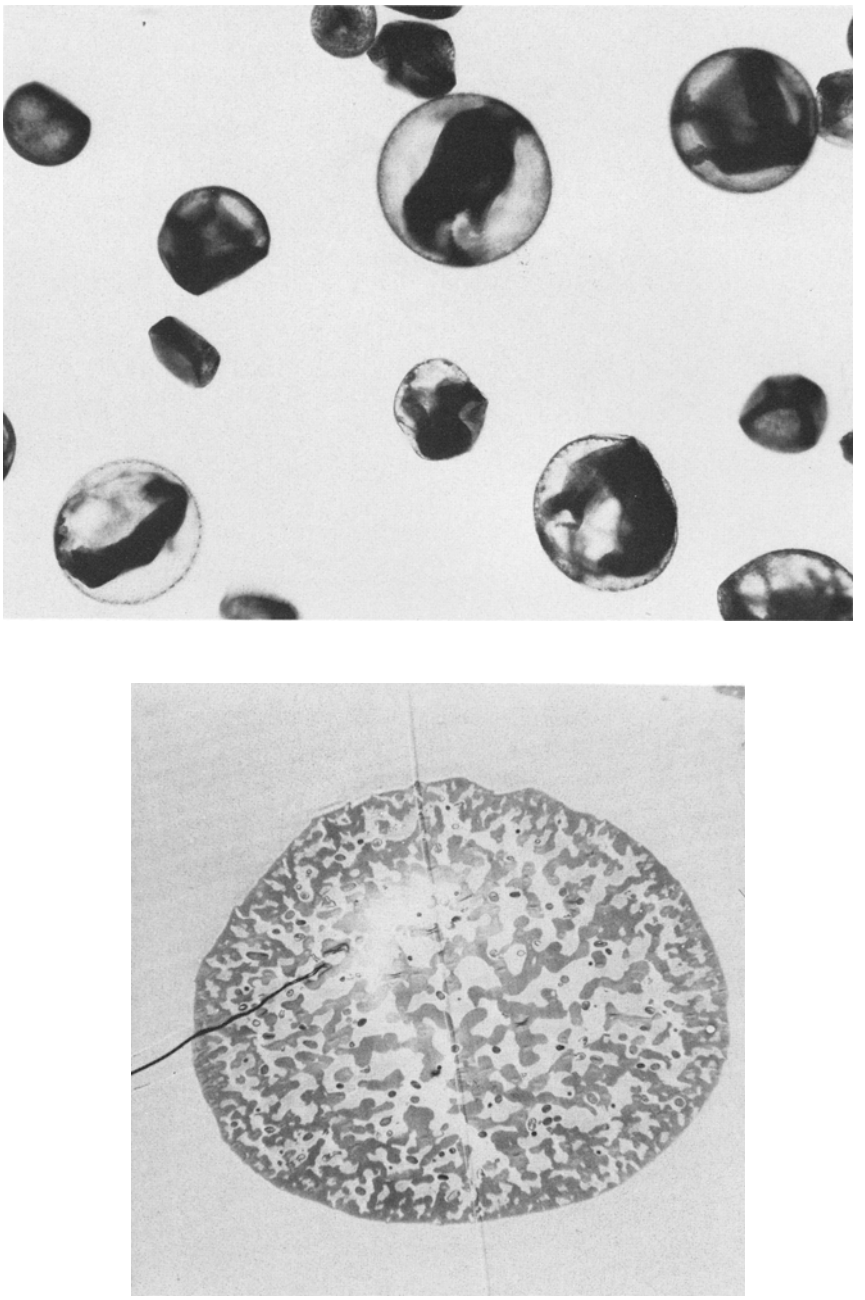

Fig. 2. Photographs of collodion microcapsules containing bacteria: (a) light microscopy (×56); (b) transmission electron microscopy (×1680).

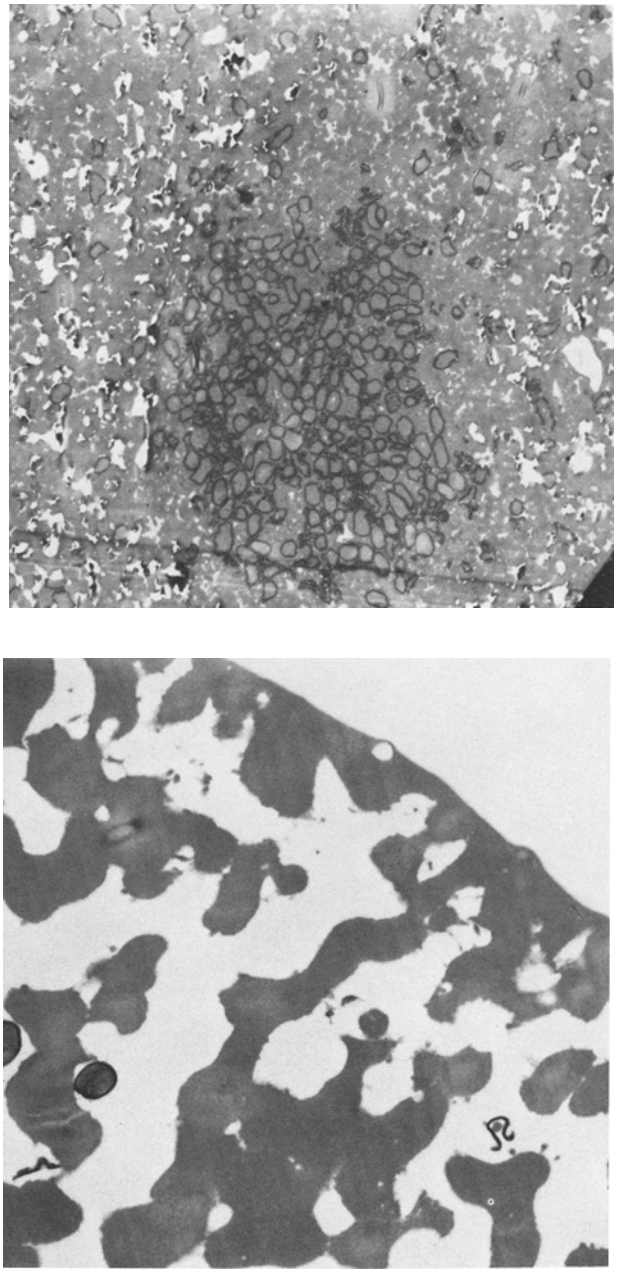

Fig. 2. (c) TEM (×8800); (d) TEM (×3200).

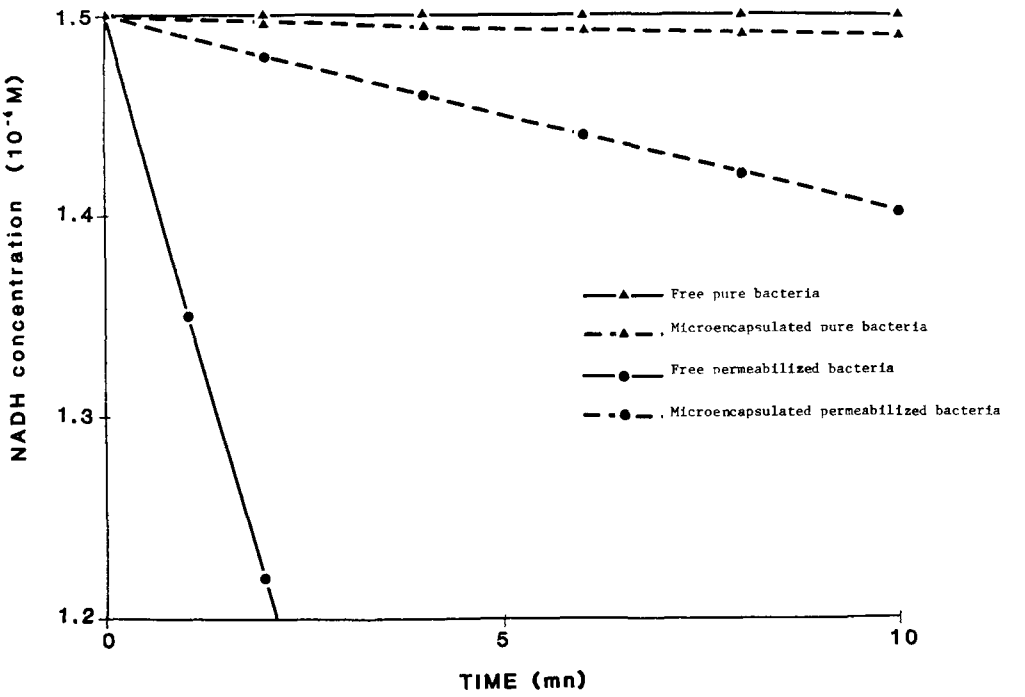

Fig. 3. Consumption of NADH with different bacterial preparations.

Feasibility of Cofactor Recycling in Solution

Figure 4 demonstrates the existence of NAD regeneration. At zero time when androsterone ($2.3 \times 10^{-4}M$), NAD ($2.9 \times 10^{-4}M$) and 3α-HSDH (0.1 mg) are mixed together, one gets an increase of NADH. Once a plateau is reached at 60 min, 20 μL of permeabilized bacteria are added, resulting in a sharp decrease in NADH. A new plateau is reached, where 33% of the previously formed NADH is not oxidized. If 6.9×10^{-7} mol of substrate is then added at $t = 2$ h, the concentration of NADH increases again, but not to the maximum original level, because of the presence of the bacteria, which continually consume some of the NADH.

Coimmobilization of Bacteria and 3α-HSDH

For this study, three experiments have been performed:

(A) Microcapsules containing both enzyme and bacteria in the presence of 2 mg of NAD, in which case recycling is possible. If no recycling occurs, the maximum theoretical con-

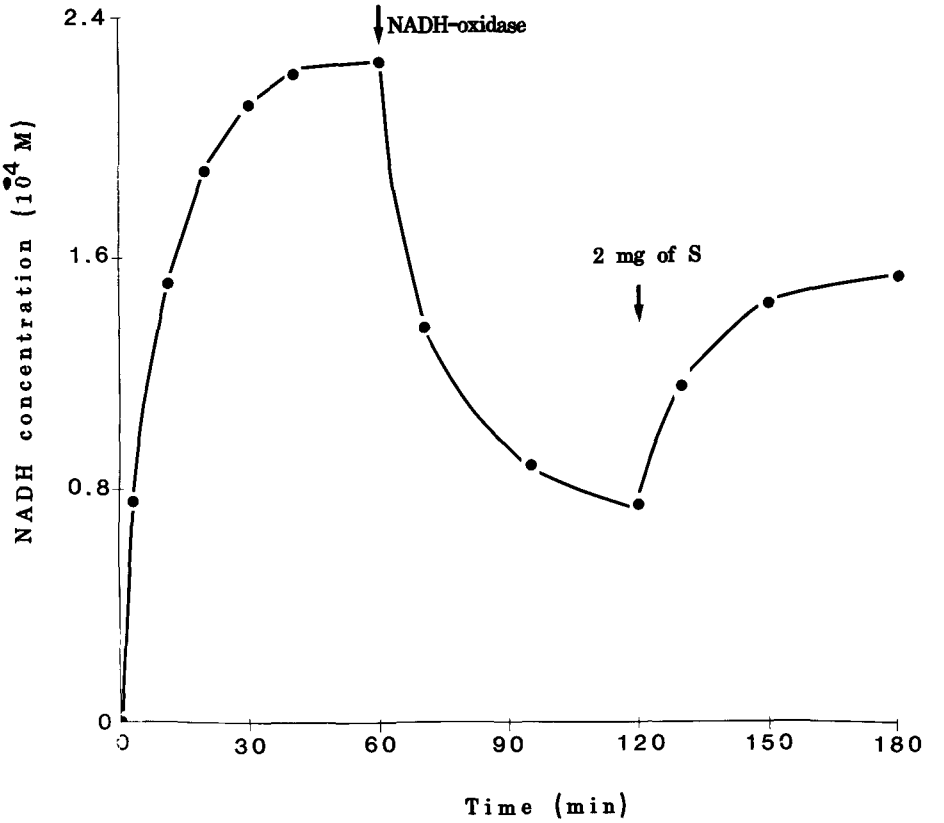

Fig. 4. Cofactor recycling in solution.

sumption of substrate possible using 2 mg of NAD is 2.85×10^{-6} mol, i.e., 40% of the original amount.

(B) Microcapsules containing only 3α-HSDH in the presence of 6 mg of NAD.

(C) Microcapsules containing only 3α-HSDH, in the presence of 2 mg of NAD.

The results are summarized in Fig. 5. The enzyme 3α-HSDH encapsulated alone, allows a consumption of 80% of the substrate, if the cofactor is supplied in sufficient amount (B). If not (C), approximatively 27% of the substrate is consumed. Working under the same conditions as (C), but with the recycling system (A), we increase the substrate consumption to 60%, which means that each molecule of NAD is utilized at least 2.2 times. This shows clear evidence of recycling when 3α-HSDH and bacteria are coimmobilized in the same microcapsules.

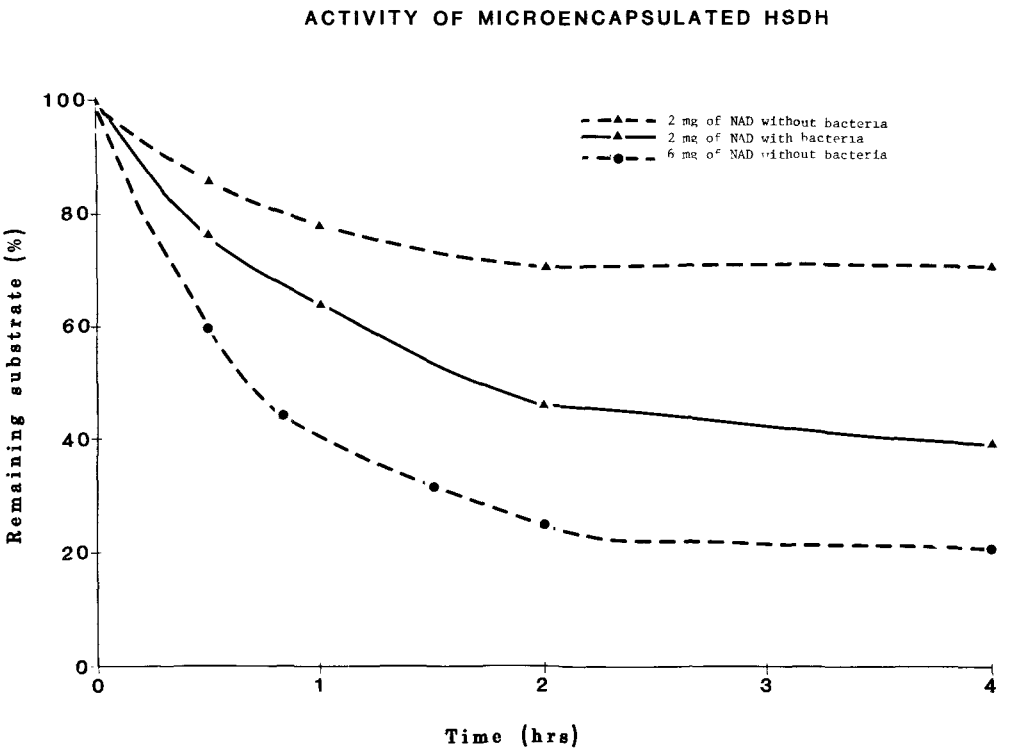

Fig. 5. Consumption of substrate with or without recycling in presence of different amounts of NAD.

Repeated Use of an Immobilized Multienzyme System in a Batch Reactor

This experiment is performed under the same conditions as outlined in "coimmobilization of bacteria and 3α-HSDH" (A). After every 2 h, 2 mg of substrate is added to the reaction chamber. Figure 6 shows the percentage of remaining substrate as a function of time.

After 10 h, one gets a total consumption of 6.30 mg of substrate, that is 2.17×10^{-5} mol. However, only 2.85×10^{-6} mol of NAD were available. These results indicate that each molecule of NAD has been used 7.6 times. We stopped the reaction after 10 h, because the very low solubility of the product was making the medium very turbid, thus preventing us from carrying out accurate readings. Future investigations will attempt to elucidate precisely what is the limiting step of this process.

CONCLUSION

An efficient bacteria has been found to oxidize NADH back to NAD so that it can be reused as a cofactor for the enzymatic conversion. Moreover, the feasibility of the recycling in microcapsules has been shown.

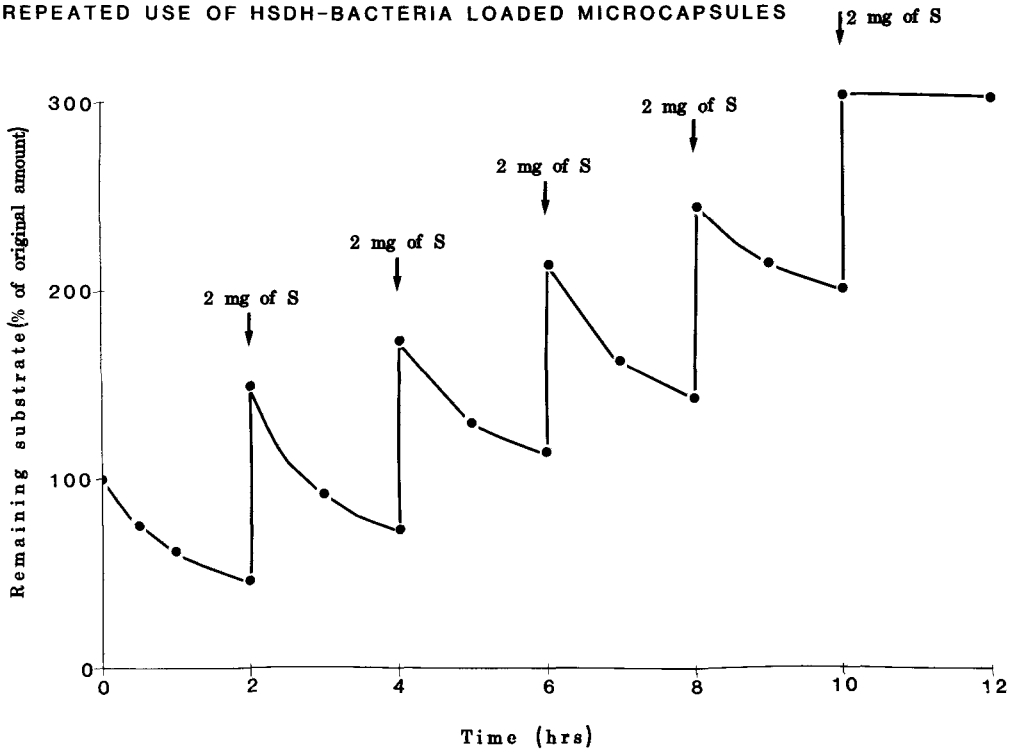

Fig. 6. Repeated use of the immobilized multienzyme system in a batch reactor.

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