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Electrochemical Reduction of Xylose to Xylitol by Whole Cells or Crude Enzyme of *Candida peltata*

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In this study, whole cells and a crude enzyme of *Candida peltata* were applied to an electrochemical bioreactor, in order to induce an increment of the reduction of xylose to xylitol. Neutral red was utilized as an electron mediator in the whole cell reactor, and a graphite-Mn(IV) electrode was used as a catalyst in the enzyme reactor in order to induce the electrochemical reduction of NAD^+ to NADH. The efficiency with which xylose was converted to xylitol in the electrochemical bioreactor was five times higher than that in the conventional bioreactor, when whole cells were employed as a biocatalyst. Meanwhile, the xylose to xylitol reduction efficiency in the enzyme reactor using the graphite-Mn (IV) electrode and NAD^+ was twice as high as that observed in the conventional bioreactor which utilized NADH as a reducing power. In order to use the graphite-Mn(IV) electrode as a catalyst for the reduction of NAD^+ to NADH, a bioelectrocatalyst was engineered, namely, oxidoreductase (e.g. xylose reductase). NAD^+ can function in this biotransformation procedure without any electron mediator or a second oxidoreductase for NAD^+/NADH recycling

Key words: electrochemical oxidoreduction, graphite-Mn(IV) electrode, neutral red, xylitol, xylose reductase

The cofactors, NADH and NADPH, are instrumental in the enzymatic reduction of xylose to xylitol via xylose reductase (Lee *et al.*, 2000). One major limitation inherent to the utilization of reductases in biochemical and chemical syntheses (Miyawaki and Yano, 1993) is the lack of a simple regeneration or recycling system for the electron-transferring cofactors (NAD^+, $NADP^+$, FAD, etc.) (Chenault and Whitesides, 1987; Wong and Whitesides, 1994; Fang and Lin, 1995; Lee *et al.*, 2003). Within this industry, two approaches have generally been applied in order to rectify this problem. When working with isolated enzymes, a second enzyme can be employed. In the case of NADH, the best approach thus far has been to use formate dehydrogenase, which catalyzes the reduction of NAD^+ to NADH, coupled with the oxidation of formate into CO_2 (Jang *et al.*, 1993; Krage *et al.*, 1996). The other approach involves the application of whole cells, using glucose, for example, as a C-source: Using this approach, the multicatalyst system of the whole cell itself is used in the process of regeneration. There are a few other electrochemical methods for cofactor regeneration which have not yet been put into general use in industrial processes, but have been tested at laboratory scales (Katz *et al.*, 1999; Alverez-Gonzales *et al.*, 2000; Chen *et al.*, 2001; Munteanu *et al.*, 2001). Park and Zeikus (1999, 2000, 2002) reported that neutral red could be electrochemically oxidized and reduced on the surface of an electrode, and that neutral red undergoes a reversible chemical oxidoreduction with NAD^+ (i.e., electrochemically-recycled NAD^+). Neutral red appears to be more advantageous for use in a whole cell bioreactor than in an enzyme reactor, because neutral red binds to the cytoplasmic membrane (Park and Zeikus, 1999). When using an enzyme reactor, the mixture of the soluble electron mediator, cofactors, and enzymes renders cofactor recycling and product purification a rather difficult proposition. In the previous study, we developed a technique for the immobilization of Mn(IV) onto a graphite electrode, and also observed that the graphite-Mn(IV) electrode reciprocally catalyzed the electrochemical oxidoreduction of NAD^+/NADH (Shin *et al.*, 2004). Recently, yeast cells have been used in the generation of bioactive compounds, including enzymes (Kim and Park, 2004) and antioxidant compounds (Cha *et al.*, 2004; Kim *et al.*, 2004).

Electrochemically-reduced neutral red can be reoxidized, along with the reduction of NAD^+ to NADH, in the cytoplasm of yeast cells. The Mn(IV) immobilized onto the graphite electrodes functions as a catalyst for the electrochemical reduction of NAD^+ to NADH within the

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enzyme reactor. In this study, we have applied a simplified regeneration system, composed of neutral red or graphite-Mn(IV) electrodes, to the whole cell or to the enzyme reactor, in order to increase the rate at which xylose can be reduced to xylitol.

Materials and Methods

Chemicals

The D-xylose, xylitol, NAD^+, and NADH used in this study were purchased from Sigma (USA). The protein standard and Bradford reagent were obtained from Bio-Rad (USA).

Catalyst

Candida peltata (NRRL Y-6888) cells were cultivated in YMP broth (Difco, USA; Yeast ext. 3 g/l, malt ext. 5 g/l and peptone 10 g/l) for 30 h at 30°C, harvested via 30 min of centrifugation at 4°C and 8,000 x g, then washed twice with 50 mM phosphate buffer (pH 6.5) and disrupted with a French press at 20,000 psi and 4°C. The cell-free extracts were prepared via centrifugation of the disrupted yeast cells at 15,000xg and 4°C for 40 min. This was used as the crude enzyme. The resting yeast cells were collected by 30 min of aseptic centrifugation at 4°C and 8,000xg, and then resuspended in a 1/100 volume of YMP broth.

Xylose reductase activity assay

The activity of xylose reductase was determined according to the methods described by Webb and Lee (1991), with some slight modifications. The concentration of NADH oxidation coupled with the reduction of xylose to xylitol was spectrophotometrically assessed at a wavelength of 340 nm. This measurement began with the addition of 2 mM NADH to a reactant containing 50 mM phosphate buffer (pH 6.5), 10 mM xylose, and 5 mg/ml of crude enzyme. The activity level was calculated in terms of the concentration of NADH oxidized per min and mg protein.

Electrode composition

The graphite-Mn(IV) anode used in the study was constructed of a mixture of 60% (w/w) fine graphite powder, 37% (w/w) inorganic binder, and 3.0% (w/w) manganese ions, according to the method utilized in the previous study (Shin *et al.*, 2004).

Porcelain membrane

A porcelain membrane was constructed by the same method as was followed in the previous study (Shin *et al.*, 2004).

Electrochemical and conventional bioreactor

The electrochemical bioreactor used in this study was designed and constructed according to the specifications established in the previous study (Shin *et al.*, 2004), but the working volume was expanded to 250 ml. A 500 ml, medium-sized bottle was used as a conventional bioreactor. The aeration port, sampling port, and gas outlet were fitted to both the electrochemical and conventional bioreactors. In the whole cell bioreactor, the graphite electrode was used as a cathode, and the platinum electrode was used as an anode, as is shown in Fig. 1A. Phosphate buffer (50 mM, pH 6.5) containing 200 μM neutral red and 100 mM xylose was employed as the basal reactant, to which the resting cells of *Candida peltata* were added aseptically immediately prior to the initiation of the reaction. During the reaction, the bioreactors were incubated

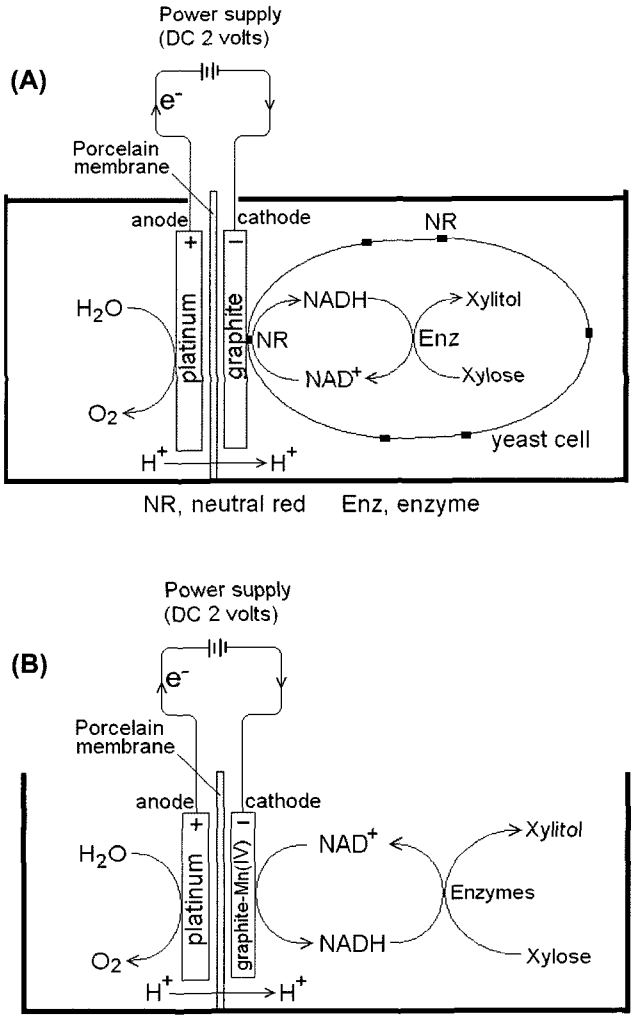

Fig. 1. Schematic structure of the electrochemical reactor for the whole cell (A) and enzyme (B) reactions. The whole cell reactor was designed as a closed system to protect against contamination during long-term incubations, but the enzyme reactor was designed as an open system, because the reaction time was less than three hours. 50 mM phosphate buffer (pH 6.5) containing substrates, cofactor and biocatalyst was used as a catholyte (reactant), and 200 mM phosphate buffer (pH 6.5) containing 200 mM NaCl was used as an anolyte. The anode and cathode were separated by a porcelain membrane.

at 30°C under microaerobic conditions, and the dissolved oxygen in the reactant was adjusted to 0.5-1.0 mg/l.

In the enzyme bioreactor, we used a graphite-Mn(IV) electrode as a catalyst for the reduction of NAD^+ to NADH, and a platinum electrode was utilized as an anode, as is shown in Fig. 1B. 50 mM phosphate buffer (pH 6.5) containing 10 mM xylose and 0.5 mM NAD^+ was used in the electrochemical bioreactor as a basal reactant, and 10 mM NADH, rather than NAD^+, was used in the conventional bioreactor. 200 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl was used as an anolyte. Prior to the beginning of the reaction, 2 volts of DC electricity were applied to the electrodes, and N_2 was sparged into the reaction mixture for 60 min, in order to remove O_2.

Analysis

The levels of D-xylose and xylitol were determined using an HPLC system (YoungLin Systems, Korea) equipped with an HPX-87H organic acid column (BioRad, USA) and an RI detector. 0.008N sulfuric acid was used as the mobile phase, and the flow rate was adjusted to 0.6 ml.

Results and Discussion

Xylose reductase activity in the crude enzyme

Xylose reductase catalyzes the reduction of xylose to xylitol, combined with the oxidation of NADH to NAD^+, which was spectrophotometrically determined at 340 nm. The crude enzyme isolated from *Candida peltata* oxidized 8.4 M NADH $min^{-1}mg\ protein^{-1}$, which represented a lesser degree of activity than that of the crude xylose reductase which was isolated from *Saccharomyces cerevisiae* (Lee *et al.*, 2003, Jang *et al.*, 2003).

Xylitol production by resting cells

Certain yeast cells have been shown to be able to reduce xylose to xylitol, along with a concomitant oxidation of NADH to NAD^+, which is again reduced to NADH along with a concomitant oxidation of xylose or another carbon source (Thestrup and Hägerdal, 1995). Under conditions in which the substrate and catalyst (yeast cell) exist in the same concentrations, xylitol generation may be proportional to the NADH/NAD^+ ratio within the cytoplasm. As is shown in Fig. 2, the production of xylitol was about five times higher in the electrochemical bioreactor than in the conventional bioreactor, but was not activated in the electrochemical reactor in the absence of neutral red. According to this result, it can be supposed that electrochemically reduced neutral red may activate the reduction of NAD^+ to NADH, thereby allowing a higher NADH/NAD^+ ratio to be maintained in the cytoplasm of *Candida peltata*. Park and Zeikus (1999, 2000) reported that neutral red immobilized in the cytoplasmic membrane of *Actinobacillus succinogenes* served to activate the electrochemical reduction of NAD^+ to NADH, by which a 20-40% increment in the generated products could be achieved. Shin *et al.* (2001) reported that the reduction of β-tetralone (ketone) to β-tetralol (alcohol) was activated when neutral red was added to the electrochemical bioreactor, using *Trichosporon capitatum* as a biocatalyst. It remains uncertain as to whether neutral red can activate the chemical reduction of NAD^+ to NADH via the same mechanism in *Candida peltata* as it does in *Actinobacillus succinogenes*. However, it can be surmised that the electrochemical reduction of xylose to xylitol is activated in *Candida peltata* on the basis of the structural and physiological similarity of *Candida peltata* to *Trichosporon*

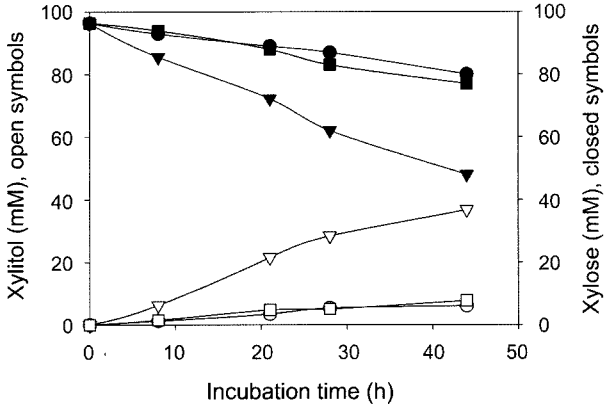

Fig. 2. Biochemical and electrochemical reduction of xylose to xylitol by resting *C. peltata* cells in the conventional bioreactor (circular symbols) and the electrochemical bioreactors, both with neutral red (triangular symbols) and without neutral red (rectangular symbols). Phosphate buffer (50 mM, pH 7.5) containing 100 mM xylose and 200 μM neutral red was used as a reactant for both the electrochemical and conventional bioreactors.

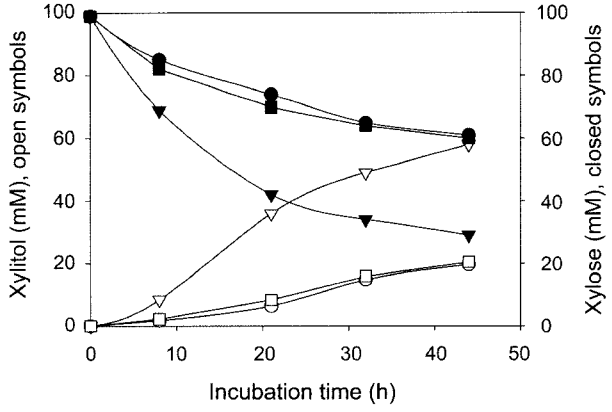

Fig. 3. Effects of glucose on the biochemical and electrochemical reduction of xylose to xylitol by resting *C. peltata* cells in the conventional bioreactor (circular symbols) and the electrochemical bioreactor, both with neutral red (triangular symbols) and without neutral red (rectangular symbols). Phosphate buffer (50 mM, pH 7.5) containing 100 mM glucose, 100 mM xylose and 200 μM neutral red was used as a reactant for both the electrochemical and conventional bioreactors.

capitatum (Carlile *et al.*, 2001). Under growth conditions in which there is no extra electron donor, such as glucose or an electrochemical reducing power, the reducing power for the reduction of NAD^+ to NADH must be generated from the xylose itself. In this case, some of xylose is oxidized concomitantly with the reduction of NAD^+ to NADH, and some of the xylose is reduced to xylitol, combined with the oxidation of NADH to NAD^+. Therefore, the amount of xylitol produced must be relatively higher in a bioreactor using glucose or electrochemical reducing power. As is shown in Fig. 3, xylitol production was increased to a higher degree in the electrochemical bioreactor with neutral red by the addition of glucose. However, this increase was not observed in the electrochemical bioreactor to which neutral red had not been added. The effects of electrochemical reducing power on xylitol production appear to be completely dependent on neutral red, but these effects do not appear to be related to glucose. This suggests that the electrochemical reduction of neutral red is coeval with the reduction of NAD^+ to NADH, and the biochemical reduction of xylose to xylitol is coeval with the oxidation of NADH to NAD^+.

Xylitol production in the enzyme bioreactor

In the previous study (Shin *et al.*, 2004), it was confirmed that an electrode modified with Mn(IV) can catalyze the oxidoreduction of NAD^+/NADH, in a fashion independent of both enzyme catalysis and electron mediators. However, the NADH reduced by electrochemical catalysis has not yet proven to be a suitable cofactor for xylose reductase. In order to determine the function of electrochemically reduced NADH, we added 0.5 mM NAD^+ and 10 mM NADH to an electrochemical bioreactor and a conventional bioreactor. As is shown in Fig. 4, 3.5 mM of xylitol was generated from 10 mM xylose in the electrochemical bioreactor, and 1.5 mM of xylitol was produced in the conventional bioreactor. However, no xylitol was generated in the conventional bioreactor to which NADH had not been added. Theoretically, xylitol generation should be proportional to NADH concentration within an enzyme reactor; however, the amount of xylitol produced was still higher than in the electrochemical bioreactor to which NADH had not been added. From this result, we obtained reasonable evidence to suggest that NAD^+ can be electrochemically reduced to NADH by a graphite-Mn (IV) electrode, and the electrochemically reduced NADH can function as a cofactor for xylose reductase, similarly to the biochemically reduced NADH. In order to evaluate the enzymatic reduction of xylose to xylitol, we employed the crude enzyme as a catalyst, a method which may be advantageous when using an industrial bioreactor, as the purification cost is quite high. Moreover, crude enzymes can be extracted from frozen bacterial or yeast cells whenever required. Recombinant proteins have been used in bioreactors as catalysts, but mass production with such a scheme would entail prohibitively high costs. The inability or expense of cofactor recycling and enzyme purification has severely limited the industrial use of oxidoreductases (Alverez-Gonzales *et al.*, 2000). According to the present method, several bioelectrocatalysts can be engineered: namely, oxidoreductase (e.g. xylose reductase) and NAD^+, both of which can be used for biotransformation without an electron mediator or a second oxidoreductase for NAD^+/NADH recycling. The xylitol yield can never, however, exceed the NADH concentration, because the rates of reduction of xylose to xylitol are proportional to the rates of NADH oxidation in the biochemical reactor. However, the xylitol yield is proportional to the recycling number of NAD^+/NADH in an electrochemical bioreactor. We are currently researching a process by which both xylitol reductase and NAD^+ can be bound onto a graphite-Mn(IV) electrode, in order to construct a bioreactor for continuous xylitol production. Also, several new methods for the electrical recycling of cofactors are currently in the development stage. These new methods might someday, perhaps, be applied in order to further improve our method for the engineering of bioelectrocatalyst.

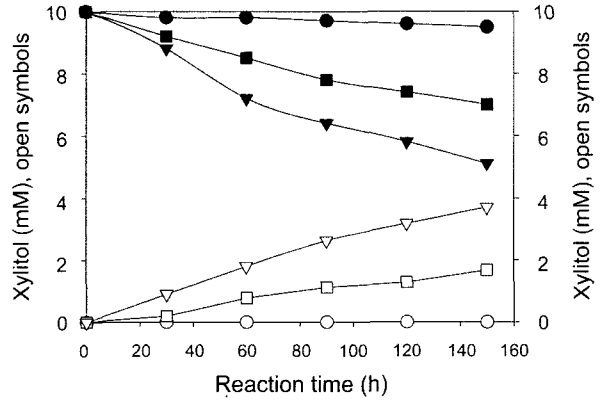

Fig. 4. Biochemical and electrochemical reduction of xylose to xylitol by the crude enzyme isolated from *C. peltata* in the conventional bioreactor (rectangular symbols) and the electrochemical bioreactor (inverted triangular symbols). Phosphate buffer (50 mM, pH 7.5) containing 10 mM xylose was used as a basal reactant. 0.5 mM NAD^+ was added to the electrochemical reactor, and 10 mM NADH was added to the conventional reactor. The control test was performed with basal reactant in the conventional reactor (circular symbol).

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