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Complete *In Vitro* Conversion of D-Xylose to Xylitol by Coupling Xylose Reductase and Formate Dehydrogenase

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Abstract Artificial coupling of one enzyme with another can provide an efficient means for the production of industrially important chemicals. Xylose reductase has been recently discovered to be useful in the reductive production of xylitol. However, a limitation of its *in vitro* or *in vivo* use is the regeneration of the cofactor NAD(P)H in the enzyme activity. In the present study, an efficient process for the production of xylitol from D-xylose was established by coupling two enzymes. A NADH-dependent xylose reductase (XR) from *Pichia stipitis* catalyzed the reduction of xylose with a stoichiometric consumption of NADH, and the resulting cofactor NAD^+ was continuously re-reduced by formate dehydrogenase (FDH) for regeneration. Using simple kinetic analyses as tools for process optimization, suitable conditions for the performance and yield of the coupled reaction were established. The optimal reaction temperature and pH were determined to be about 30°C and 7.0, respectively. Formate, as a substrate of FDH, affected the yield and cofactor regeneration, and was, therefore, adjusted to a concentration of 20 mM. When the total activity of FDH was about 1.8-fold higher than that of XR, the performance was better than that by any other activity ratios. As expected, there were no distinct differences in the conversion yields of reactions, when supplied with the oxidized form NAD^+ instead of the reduced form NADH, as a starting cofactor for regeneration. Under these conditions, a complete conversion (>99%) could be readily obtained from a small-scale batch reaction.

Key words: Xylitol, xylose reductase, formate dehydrogenase, cofactor regeneration

Xylitol, a five-carbon sugar alcohol, occurs widely in nature as an ingredient of various fruits, vegetables, mushrooms, and microorganisms, and it is also found as a normal intermediate of carbohydrate metabolism in various eukaryotic cells, especially human [13]. Xylitol is currently used as an alternative sweetener in the food industry due to its sweetening power, which is comparable to that of sucrose, and it is recommended for treating diabetic conditions and preventing dental caries [5, 17, 20]. Therefore, a method for recovering a high amount of xylitol directly from natural sources or an efficient process for large-scale production would be economically valuable for various applications.

As the reductive product from D-xylose *in vivo*, xylitol has been produced routinely by a fermentation step or enzymatic process, commonly all within the yeast cells [8, 19]. These biocatalytic processes have many advantages over a chemical process. For example, the chemical hydrogenation of D-xylose using a metal catalyst under extreme conditions was accompanied with inevitable problems, such as the high cost of downstream processing and disposal of wastewater [3]. The reason for this was probably due to the complexity of the natural resources of lignocellulosic biomass, cellulose and hemicellulose, when they were used as the initial resource for xylitol production. Microbial production of xylitol, therefore, became a more attractive process since its downstream process was known to be cheaper, and a high cell density culture and thus high yield in productivity were also readily obtainable [7, 10]. Among the selected candidates, yeast cells, particularly the genus *Candida*, was the most prevalent and best xylitol producer. Current industrial production of xylitol using such a strain has been reported to a yield approximately 80% [14].

The key steps for xylitol metabolism in yeast cells, including *Pichia* and *Candida*, are represented by a successive reaction mediated by NAD(P)H-dependent xylose reductase (XR, EC 1.1.1.21) and NAD^+-dependent xylitol dehydrogenase (XDH, EC 1.1.1.14). The former enzyme reduces D-xylose to xylitol, and it is further metabolized to D-xylulose by the enzyme XDH. Therefore, there are two possible strategies

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that could enhance xylitol yields in these strains. One is the introduction of a XR gene into a producer, permitting a relatively high expression of the enzyme and thus high productivity. The other is the construction of a XDH-defective mutant, thereby blocking the further conversion of xylitol to D-xylulose. In this context, a metabolically engineered yeast cell containing the amplified XR gene [2, 9, 11] and a XDH defective mutant of the yeast *Pichia stipitis* [10] have been developed to produce xylitol with a yield of nearly 100%, but unexpectedly both strains showed relatively lower volumetric productivities and final xylitol concentrations than the wild-type cells. The reason for this is probably that, during a prolonged fermentation or incubation time for enzymatic conversion, a whole or high conversion of D-xylose to xylitol by XR could be possible only with a continuous supply or regeneration of a cofactor, NAD(P)H, regardless of whether the reactions are performed *in vitro* or *in vivo*. A previous report showed that there may be high xylitol productivity in a coupled reaction with cofactor regeneration [15]. However, detailed information describing the reactions, both single and coupled, has not been provided.

This study attempted a coupled reaction that employed an NADH-consuming XR of *P. stipitis* and an NADH-producing formate dehydrogenase (FDH, EC 1.2.1.2) of *Candida boidinii* for cofactor regeneration [12, 18]. Thereby, reusable enzyme activities for continuous reaction were achieved. The XR and FDH activity in both individual and coupled reactions were further analyzed in terms of optimum pH, temperature stability, cofactor, and enzyme loading. A small-scale reaction also demonstrated the usefulness of this coupled reaction as a practical method for the enzymatic production of xylitol by complete conversion with a theoretical yield of 100%.

MATERIALS AND METHODS

Chemical

D-Xylose, xylitol, formic acid, NAD(P)$^+$, and NAD(P)H were purchased from Sigma (St. Louis, MO, U.S.A.). Acrylamide stock (30%) and protein assay solutions were purchased from Bio-Rad (Richmond, CA, U.S.A). All other chemicals and solvents used were of analytical grade.

Microorganisms and Culture Conditions

To analyze and evaluate a coupled reaction for performance and validity, the mutant strain *P. stipitis* PXM-4, that lacked the XDH activity and thus blocked the further metabolism to D-xylulose, was used as the source of the enzyme XR [10], and a strain *C. boidinii* was used as the source of the enzyme FDH [18]. The strain PXM-4 was routinely grown and periodically transferred at 30°C to YPD medium (10 g/l yeast extract, 10 g/l bacto peptone, and 20 g/l D-glucose), and *C. boidinii* was grown on YPDF medium (10 g/l yeast extract, 10 g/l bacto peptone, 20 g/l D-glucose, and 20 g/l formic acid). Seed cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of the respective medium for each variety of cells. To obtain high cell masses as enzyme sources, a main culture medium of PXM-4 was prepared as follows: 5 g/l yeast extract, 5 g/l bacto peptone, 5 g/l KH_2PO_4, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l $(NH_4)_2SO_4$, 20 g/l gluconic acid, and 20 g/l D-xylose. The main culture was carried out in a 2.5-l bioreactor (KoBioTech, Incheon, Korea) with 1.5 l working volume. The agitation speed and aeration rate were set at 250 rpm and 1 vvm, respectively. The medium for the main culture of *C. boidinii* consisted of 10 g/l yeast extract, 10 g/l bacto peptone, 30 g/l formic acid, and 30 g/l D-glucose, and was agitated at 500 rpm and aerated at 1.5 vvm. Cell growths of both cultures were carried out at 30°C, and foams were controlled by the addition of an antifoam agent.

Preparation of Cell Extracts

To obtain the crude extracts for analyzing the reaction parameters of cell-free conversion, the two yeast strains, expressing the XR and FDH activity, were cultivated in a 1.5 l liquid medium at 30°C for 18–24 h. The cells were harvested by centrifugation at 3,000 ×g for 30 min, and the resulting pellets were washed twice with a buffer (50 mM phosphate, pH 7.0). After re-centrifugation, the cells were resuspended in an appropriate volume of the same buffer containing EDTA (1 mM), lyticase (0.5 mg/ml, 420 unit/mg), and protease inhibitor cocktail solution (Sigma), and then disrupted by sonication. Cell debris was removed by centrifugation at 8,000 ×g for 30 min. The supernatant was further clarified and partly fractionated by applying it onto a desalting column PD-10 (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting solutions were used for enzyme activity and protein expression analyses, and then stored at 4°C for further analyses and conversion experiments.

pH and Temperature Dependency

To determine the optimal pH, the reaction mixture was incubated at 30°C in the following buffers: glycine-HCl (pH 3.0), citrate-NaOH (pH 4.0–5.0), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 10.0). The pH stability was also investigated by determining the residual activity after preincubation at 30°C for 2 h in the above buffers. To determine the optimal temperature, the reaction was conducted at various temperatures, ranging from 10 to 60°C, in potassium phosphate buffer (50 mM, pH 7.0). The thermal stability was determined by measuring the remaining activity after preincubation at the indicated temperatures for 2 h.

Enzyme Assay

For the analyses of XR and FDH activities, the reactions were continuously monitored by measuring increase or

decrease of absorbance at 340 nm in a spectrophotometer. The standard assay mixtures consisted of 50 mM phosphate (pH 7.0), crude enzyme extracts (10 mg protein), cofactor (NAD^+ or NADH, 100 mM), and substrate (1 M). The reaction mixtures were incubated at 30°C for appropriate time intervals. The reaction mixtures were also analyzed using high performance liquid chromatography (Waters, Miliford, U.S.A.). As for the analyses of xylose and xylitol, the Carbohydrate Analysis column (Waters) equilibrated with 85% acetonitrile and 15% DDW was used at the constant flow (2.0 ml/min), and the eluent was monitored using a RI detector (Waters). The concentration of formate was also determined by HPLC (UV detector, 210 nm) using an Aminex column (Bio-Rad, HPX-87H). Sulfuric acid (5 mM) was used at a flow rate of 0.6 ml/min at 40°C as the mobile phase.

One unit of XR activity was defined as the amount of enzyme to produce one μmol of xylitol from D-xylose per minute under specified conditions. The activity unit of FDH was also the amount of enzyme required to reduce one mol of formate under the same conditions. Protein concentration was measured by using a protein assay solution (Bio-Rad).

Cofactor Specificity and Kinetic Constant

The cofactor specificities of two enzymes were determined at 30°C for 1 h with constant shaking. The enzyme reaction was carried out with the desalted crude extracts (10 mg) in 1 ml of reaction mixture containing 100 mM cofactor (NAD^+ or NADH) under standard assay conditions. The kinetic constants, K_m and V_{max}, were also determined using various substrate concentrations, ranging from 0.001 to 25 mM (formate up to 100 mM), under specified conditions. A decrease in the concentration of formate and NADH or an increase of xylitol and NAD^+ in the reaction was analyzed according to the procedures described above, using either HPLC or a spectrophotometer. All assays were carried out in triplet and mean values were established.

Coupling Reaction

The cell-free conversions of substrate with crude extracts were performed at 30°C and 250 rpm shaker in a total 1 ml volume containing 10- 100 mM xylose, 20- 50 mM formate, and 10 mM NAD(H) under specified conditions. Fractionated crude cell extracts (10 mg protein or 55 enzyme units) were pooled and added to the reaction vial. The reaction products were analyzed using HPLC. The protein levels of XR and FDH enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pH and temperature dependencies of the coupled reaction were determined according to the procedures described above.

To determine the optimal ratio between the two enzymes and to evaluate the apparent kinetic parameters obtained using crude extracts, the coupled reaction was conducted under various activity ratios. At a 1:1 ratio, activities between the two enzymes were adjusted to an identical unit (1.23 U/ml), while the cofactor NADH was kept at a constant level (10 mM) for all attempted reactions.

RESULTS AND DISCUSSION

Batch Fermentation for Crude Enzyme Preparation

To prepare the crude enzyme solutions for activity analyses and conversion experiments, the strain PXM-4 was selected as a potential candidate of an XR producer. This choice was based on the previous result that the XDH-defective PXM-4 was suitable in the preparation of the XR solution [10]. The XDH-defective mutant strain PXM-4 was unable to utilize D-xylose as a carbon source, hence, gluconic acid was alternatively supplemented as a carbon source, because it did not tend to repress cell growth and enzyme production even at high concentrations [10]. As shown in Fig. 1, gluconic acid was gradually consumed at the rate of about 0.14 g/h and consequently exhausted at the completion of fermentation. When 20 g/l of xylose was supplemented together with gluconic acid as an inducer, the trend in cell growth was maintained for 70 h. In these conditions, the highest activity of XR was detected in the early stage of the cell growth (Fig. 1).

As a possible source for FDH activity, the strain *C. boidinii* was chosen because of its relatively high preference for the cofactor NAD^+. A preliminary formulated medium was also used to cultivate the strain *C. boidinii* in the presence of formic acid (30 g/l) as an activity inducer for

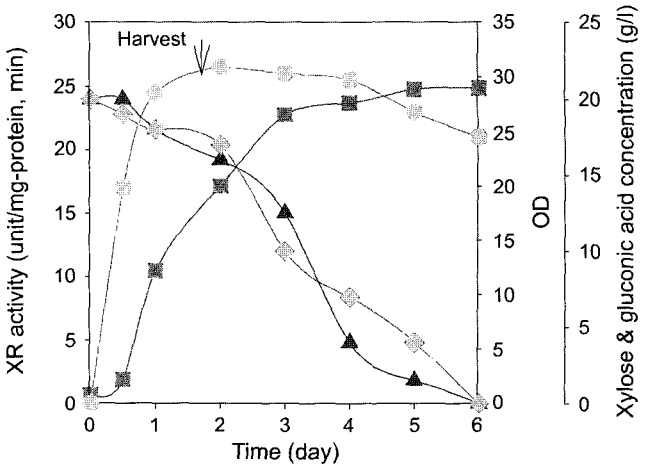

Fig. 1. Batch fermentation profile of PXM-4 for the preparation of cell-free extract.

Cell growth (■), XR activity (●), xylose (▲), and gluconic acid (◆) concentrations were monitored, when xylose (20 g/l) and gluconic acid (20 g/l) were supplemented as an inducer and carbon source, respectively. The cells were harvested at the time indicated by the arrow.

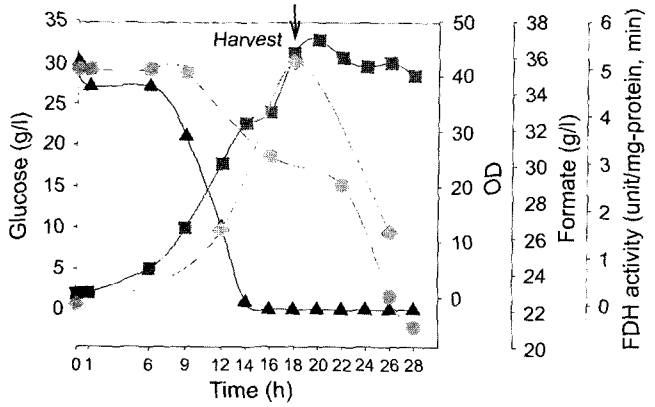

Fig. 2. Batch fermentation profile of *C. boidinii* for the preparation of cell-free extract.
Cell growth (■), FDH activity (◆), glucose (▲) and formate (●) concentrations were monitored, when glucose (30 g/l) and formate (30 g/l) were fed with preculture. The cells were harvested at the time indicated by the arrow, based on the highest specific activity.

FDH. As shown in Fig. 2, the cells were grown rapidly and reached maximum amount within 20 h when 30 g/l of glucose was used as the carbon source. In this case, the FDH activity steadily increased as the glucose concentration decreased and the activity reached a maximum when maximum growth was reached, although half of the formic acid remained still unconsumed. This is a typical trend in the growth-associated enzyme production, when catabolic repression exists. In particular, initial growth and activity were not severely affected by formic acid supplemented when seeded. As for the induction of the FDH activity, there has been a report of a relatively high level of enzyme expression, using methanol as an inducer and/or carbon source [18]. This possibility was also tested under the reported conditions, but the final cell mass of this test resulted in a quite low level (<5%) compared to the result obtained in the present study, nevertheless a slight (1.5-2.0 folds) yet distinct increase in the activity of the enzyme was observed.

Preparation of Crude Enzyme Solutions

When sampled and analyzed during the fermentation, the FDH activity was found to correlate well with the cell growth, indicating enzyme to be growth-associated, whereas the XR activity showed an explosive induction in the early stage of fermentation. To further confirm the activity of whole cell enzymes at the protein levels, the crude extracts were subsequently prepared and assayed, and the resulting products were analyzed by HPLC or spectrophotometry. The activities between whole cell and crude extract were well correlated, as expected. Cells were then harvested from 1.5 l of each culture broth at the indicated times in Figs. 1 and 2. During analyses using SDS/PAE, marginal activities (<15%) were discovered in the cell pellets of both extracts.

It is generally known that considerable reactions mediated by reductases and/or dehydrogenases are frequently detected in the crude extract, although a cofactor NAD^+ or NADH is not added exogenously. These phenomena are probably due to the presence of a significant level of innate cofactors and metabolites. To exclude interference with the coupled reaction by these natural contaminants, the crude extracts were fractionated to render the solution free of any interfering substances. For a simple fractionation and reproducibility, attempts were made to find a simple step that could be applied to both enzyme solutions, because the viscosity, ionic strength, metabolites, and protein concentrations were somewhat different in both cases. As an approach, desalting was conducted. Thus, the crude extracts were applied to a PD-10 column, and the resulting fractions with their expected activities were pooled and subjected to enzyme assays and SDS/PAGE analyses. After this step, the crude extracts of PXM-4 showed the XR activity to be about 56 U/mg-protein with no activity on formate and xylitol, thus confirming the solution was without any FDH and XDH activities. The crude extract of *C. boidinii* was also fractionated under identical conditions, and exhibited a distinct activity on formate (13 U/mg protein), without any activity on xylose and xylitol. The resulting solutions were used to characterize and optimize the coupled reaction without any further fractionation, because the main focus of this work was to evaluate a coupled reaction with crude extracts. As mentioned above, each solution only acted on its respective substrate, when its cofactor was supplied exogenously.

Reaction Properties of Individual Enzymes XR and FDH

These enzyme preparations were primarily investigated for their favorable reaction conditions. The cofactor specificities of the two enzymes were first examined, as they were principal factors in regeneration in a coupled reaction. As expected, the XR of PXM-4 showed about 1.7-fold higher activity with NADH (57 units) than with NADPH (34 units) as a cofactor. The activity of FDH (12 units) absolutely depended on the cofactor NAD^+, and there was no detectable activity in the presence of $NADP^+$. Additionally, considerable differences in the cofactor dependencies with either a low (1 mM) or high (20 mM) concentration were observed, and there were no inhibitions on XR and FDH activities by these cofactors.

As observed in other cases, it is generally known that favorable conversion by a coupled reaction is severely retarded or inhibited, unless the optimal conditions are rather overlapped, especially for pH and temperature [6]. The activity dependency on pH and temperature were thus determined for each case. Unexpectedly, the two enzymes, XR and FDH, showed similar optima at temperature of 30°C and pH of 7.0 (Fig. 3), thereby allowing two reactions

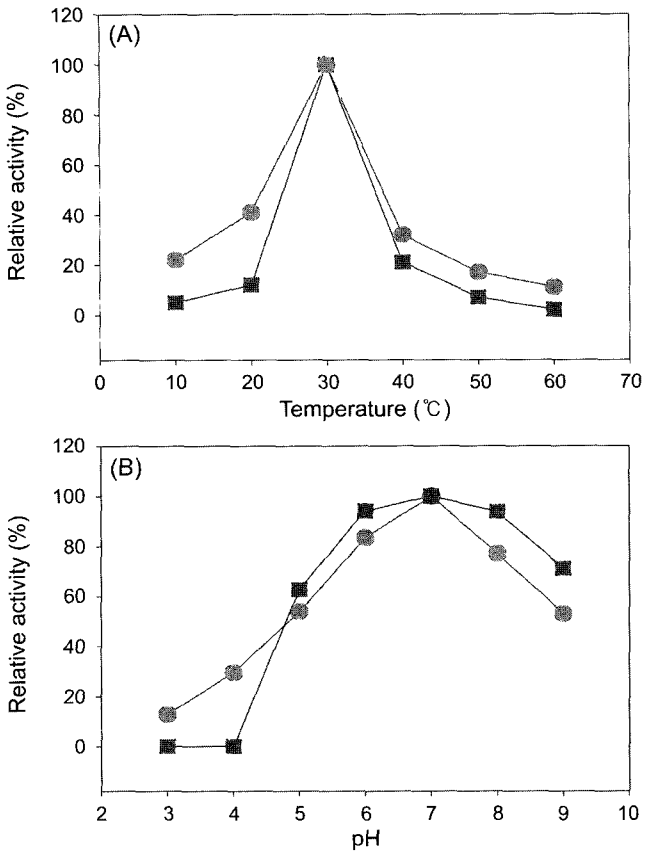

Fig. 3. Effects of temperature (A) and pH (B) on the activity of XR (■) and FDH (●).
The enzyme solutions were assayed in duplicate at the specified pH and temperature for 1 h.

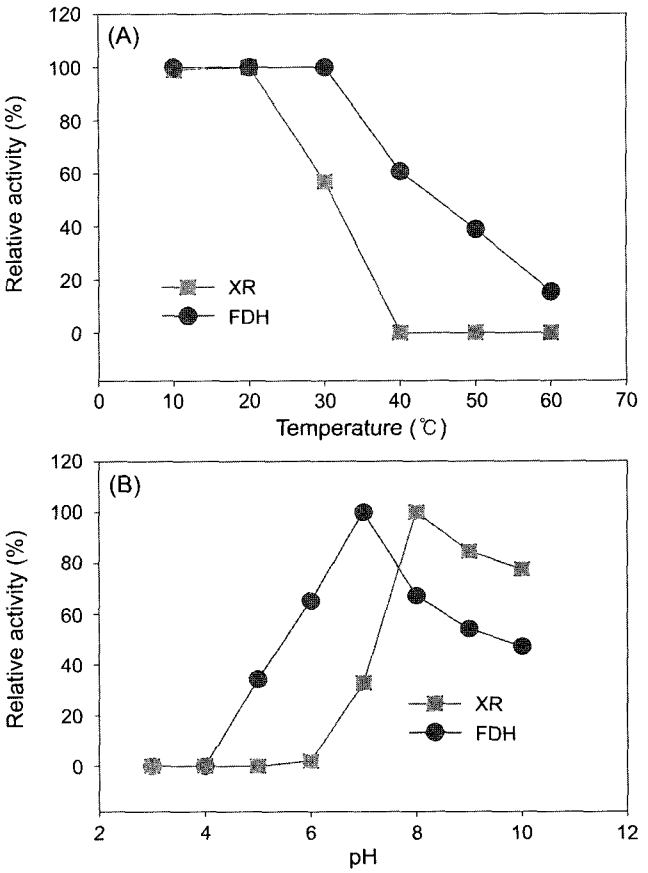

Fig. 4. Effects of temperature (A) and pH (B) on the stability of XR (■) and FDH (●).
To determine the stability, the enzyme solutions were preincubated at the specified pH and temperature for 2 h, and then assayed under standard assay conditions.

to proceed under similar conditions. The activity of XR was maintained in a stable state for more than 2 h at 30°C, but decreased to half of this level after preincubation at 40°C for 2 h (Fig. 4A). Under identical conditions, FDH rapidly lost activity within 2 h at 40°C, retaining only 10% of the initial activity. Both enzymes showed a similar trend in pH stability. When preincubated at the indicated pH for 2 h (Fig. 4B), both enzymes were found to be highly sensitive to the acidic range of pH below 7.0, but relatively stable in the alkaline pH range.

Under conditions favorable for both XR and FDH activities, the reaction constants K_m and V_{max} were determined: The reaction profiles of the two enzymes followed and expressed as a typical Michaelis-Menten equation, and their kinetic constants were readily calculated from the double reciprocal Lineweaver-Burk plot (Table 1). The apparent kinetic parameters (K_m) of XR and FDH for each substrate, xylose and formate, were about 0.28 and 0.09 mM, respectively, and the catalytic efficiency (V_{max}/K_m) was calculated to be about 19.1 and 1.3, respectively. In regard to the cofactor affinity, FDH showed 1.8-fold higher affinity to its cofactor NAD^+ than that of XR to NADH and also 2.7-fold higher catalytic efficiency than that of XR. These results strongly suggest that an appropriate ratio between the activities of the two enzymes should be precisely controlled so as to avoid depletion of the cofactor and also to ensure the continuous conversion of xylose to xylitol. There is a possibility, however, that the kinetic parameters obtained here may be somewhat different from the intrinsic values of the purified enzymes, and further

Table 1. Kinetic constants of XR and FDH.

Substrate	K_m[a] (mM)	V_{max} (mM/mg protein/min)	V_{max}/K_m (mg protein/min)
D-Xylose	0.29	5.45	19.1
NADH	0.39	7.35	18.9
Formate	87.5	112	1.28
NAD^+	0.22	11.4	50.7

[a]The kinetic constants were obtained by the experimental procedures described in Materials and Methods. Each experiment was conducted in duplicate, and the mean value is presented.

analysis is in need. From a practical standpoint, the use of crude enzymes is more preferable for biotransformation than the use of purified enzyme because of its lower cost. Thus, the kinetic parameters obtained with crude extracts appear to be more meaningful for practical application.

Cell-Free Coupling Reaction between Two Enzymes

The XR from PXM-4 and FDH from *C. boidinii* had considerable differences in each optimal reaction conditions, such as catalytic efficiency, pH, and temperature stability. The effects of these differences on the coupled reaction were examined, and the conditions were optimized in terms of conversion yield and cofactor regeneration. The initial rate of the coupled reaction was slightly increased with increasing temperature and pH, but rapid deactivation of FDH and modification of cofactor (oxidation) occurred at temperature and pH above 40°C and 8.5, respectively. The optimal reaction temperature and pH of the coupled reaction were determined to be 30°C and 7.0, respectively.

The concentration of formate supplemented as a substrate for cofactor regeneration has been considered to be an important factor, due to its inhibitory effect on the coupled partner XR [15]. To determine a formate concentration at which the XR activity was neither inhibited nor the cofactor generation was rate-limited, the reaction rates and product yields were compared using various concentrations of formate (1-100 mM) in the coupling reaction. Each enzyme activity was adjusted to be equivalent in the reaction mixture (5 U/mg protein). The results showed that the product yield and initial activity steadily increased with increasing formate concentration up to about 20 mM, however, no further increase but rather considerable inhibition was observed at high concentration up to 100 mM. This result implied that the inhibition of the coupled enzyme XR by the formate was negligible under the reaction condition, although a previous work reported that a formate concentration of 50 mM inhibited XR activity more than 80%.

When both enzymes were used, the reaction rate of each enzyme should be well balanced to have an efficient process. To determine the optimal ratio of the two enzymes and to evaluate apparent kinetic parameters obtained with crude extracts, the coupled reaction was conducted under various activity ratios (Fig. 5). All coupled reactions with varied activity ratios had a higher xylitol production level than the control reaction without the coupled FDH. In particular, xylitol concentration resulting from an activity ratio of 1:1.75 (XR:FDH) accumulated up to 2.5 mM, which was a value close to the theoretical yield (~100%). As observed in Fig. 5, the FDH-mediated NADH regeneration profoundly affected the coupled reaction, thereby limiting the reductive production of xylitol.

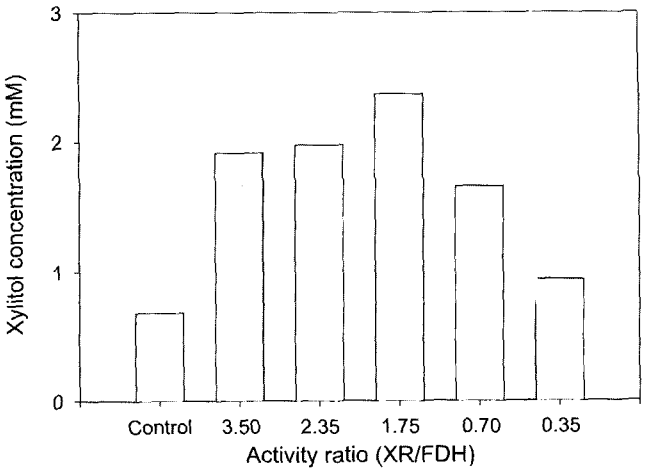

Fig. 5. Dependency of xylitol production on the activity ratio of XR and FDH.
The XR activity was fixed at 1.23 U/ml and the FDH activity was varied. All experiments were carried out in duplicate and average values are presented.

When the coupled reactions were also carried out in the presence of NAD^+ as a cofactor instead of NADH, the final yields of xylitol were also similar to that with NADH as a cofactor, although it is possible that the initial reaction with NADH might be faster than that with NAD^+, and this remains to be elucidated. When the activities of XR and FDH were fixed at a ratio of 1:1.75 in the reaction mixture, there were similar yields (>98%) of xylitol, which were shown in the reactions performed with either NAD^+, NADH, or NADH and NAD^+ (total 10 mM) as cofactors (Fig. 6). Therefore, as expected, the use of a coupled strategy for a complete conversion of xylose to xylitol will be a possible

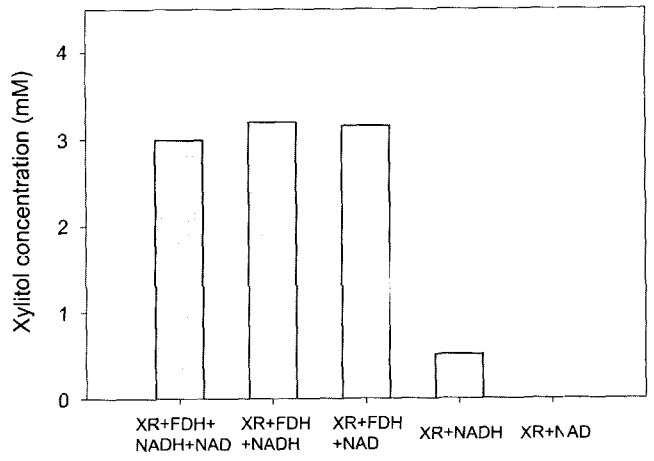

Fig. 6. Xylitol production using a coupling reaction in the presence of various cofactors.
The XR and FDH activities were fixed at an optimal ratio (1:1.75), and an appropriate cofactor (10 mM) was added exogenously. All experiments were carried out in duplicate.

strategy for further progress, although the large-scale conversion and reuse of an enzyme was not attempted here.

From an economic standpoint, a complete conversion of a substrate to the corresponding product is desirable. As a commercially valuable product, xylitol is one of the major sweeteners and is currently produced by a fermentation step or enzymatic conversion by a batch or fed-batch process with about 80% yield [1, 19]. Among the strains reported so far, the yeast strain *Pichia* or *Candida* would appear to be better candidates for xylitol production, due to their nontoxicity to humans and higher activity of XR [19]. Therefore, intensive efforts have been made to improve xylitol production by strain development or high-cell density cultures [4, 8]. As mentioned above, a yield lower than 80% has been reported, although the factors affecting this productivity have intensively been considered. The main factor is the depletion of the cofactor NAD(P)H. Accordingly, the current study demonstrates that a complete conversion of xylose to xylitol by a coupled reaction can be successfully applied to the enzymatic production of xylitol, either *in vitro*, or *in vivo*, if the enzyme FDH is successfully introduced into the XDH-defective strain PXM-4. These possibilities are partly supported by a previous report that xylitol production was enhanced by introduction of a cofactor regeneration step into the enzymatic process [15]. This report described a pH-controlled fed-batch process for cofactor regeneration, but detailed information of the conditions for complete conversion was not reported. In conclusion, although the enzymes (XR and FDH) used here are rather impractical due to their relatively low activity and stability, the current results will nevertheless serve as a basis for establishing a fully enzymatic step for the complete conversion of xylose to xylitol. Finally, it should be noted that an immobilization of two enzymes in an entrapping or absorption matrix gives a better coupling strategy [16], because it will result in a reusable or stabilized enzyme for the coupled reaction.

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