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Utilization of Xylitol Dehydrogenase in a Combined Microbial/Enzymatic Process for Production of Xylitol from D-Glucose

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Abstract

The production of xylitol from D-glucose occurs through a three-step process in which D-arabitol and D-xylulose are formed as the first and second intermediate product, respectively, and both are obtained via microbial bioconversion reactions. Catalytic hydrogenation of D-xylulose yields xylitol; however, it is contaminated with D-arabitol. The aim of this study was to increase the stereoselectivity of the D-xylulose reduction step by using enzymatic catalysis. Recombinant xylitol dehydrogenase from the yeast *Galactocandida mastotermitis* was employed to catalyze xylitol formation from D-xylulose in an NADH-dependent reaction, and coenzyme regeneration was achieved by means of formate dehydrogenase–catalyzed oxidation of formate into carbon dioxide. The xylitol yield from D-xylulose was close to 100%. Optimal productivity was found for initial coenzyme concentrations of between 0.5 and 0.75 m*M*. In the presence of 0.30 *M* (45 g/L) D-xylulose and 2000 U/L of both dehydrogenases, exhaustive substrate turnover was achieved typically in a 4-h reaction time. The enzymes were recovered after the reaction in yields of approx 90% by means of ultrafiltration and could be reused for up to six cycles of D-xylulose reduction. The advantages of incorporating the enzyme-catalyzed step in a process for producing xylitol from D-glucose are discussed, and strategies for downstream processing are proposed by which the observed coenzyme turnover number of approx 600 could be increased significantly.

Index Entries: Xylitol; coenzyme regeneration; optimization; biocatalysis.

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Introduction

Xylitol is a five-carbon polyalcohol that is found naturally in fruits and vegetables. It has a sweetening power similar to that of sucrose. This and a number of unique properties clearly distinct from those of other polyols make xylitol a very useful food ingredient and have important commercial implications. Xylitol is reported to display a dental caries–preventing effect. Because of its negative heat of dissolution, it provides a feeling of vaporization in the oral cavity and imparts a cool and fresh sensation on consumption. Therefore, xylitol is commonly added in combination with D-mannitol and D-sorbitol to sugarfree chewing gum, candy and the like. Xylitol metabolism in humans is insulinin-dependent. Combined with D-fructose, xylitol is thus a recommended sweetener in the nutrition of diabetic consumers *(1)*.

Most processes of xylitol production utilize D-xylose as the starting material. The transformation into xylitol is chemically very simple and selective and is achieved industrially by catalytic hydrogenation *(1)*. Biotechnologic alternatives to the chemical synthesis of xylitol from D-xylose have been proposed *(1–5)*. D-Xylose is abundant and renewable; however, it is difficult to obtain as a pure and defined chemical from the hemicellulose fraction of plant biomass. Pretreatment and hydrolysis of lignocellulose yields a multicomponent product mixture that in addition to D-xylose contains other reducing sugars, acids, and various products of decomposition of lignin and sugars. Therefore, extensive purification is required either as part of the upstream processing of the substrate D-xylose to remove compounds that might interfere or inhibit the actual conversion, or during the isolation of the product xylitol. Consequently, the maximum yield of xylitol from hemicellulose is 50% (per weight) of the pentosan present in the raw material, and the cost of xylitol production is high compared with production costs of other polyalcohols such as D-sorbitol or D-mannitol. Xylitol cost is, therefore, the major factor limiting the range of current applications of this polyol in food and chemical technology *(3)*.

The conversion of starch into D-glucose is technically much simpler and by far less costly than the conversion of hemicellulose into D-xylose. Considering the obvious technologic and economic advantages of D-glucose over D-xylose, a process for xylitol production that starts from D-glucose might be an interesting alternative to the classic process which employs D-xylose as the substrate. It has been known for at least 30 yr that D-glucose can be converted microbially into xylitol via D-arabitol and D-xylulose *(6)*. Figure 1 illustrates this stepwise process schematically. The initial conversion of D-glucose into D-arabitol occurs during aerobic growth on D-glucose of a number of osmophilic yeast strains of the genera *Pichia*, *Candida*, and others *(6,7)*. D-arabitol is produced presumably via the pentose phosphate pathway *(8)*, and D-arabitol dehydrogenase is thought to be responsible for D-arabitol formation by catalyzing the stereospecific reduction of D-ribulose *(8)*. Since D-arabitol is a byproduct of yeast cell mass production, the typical

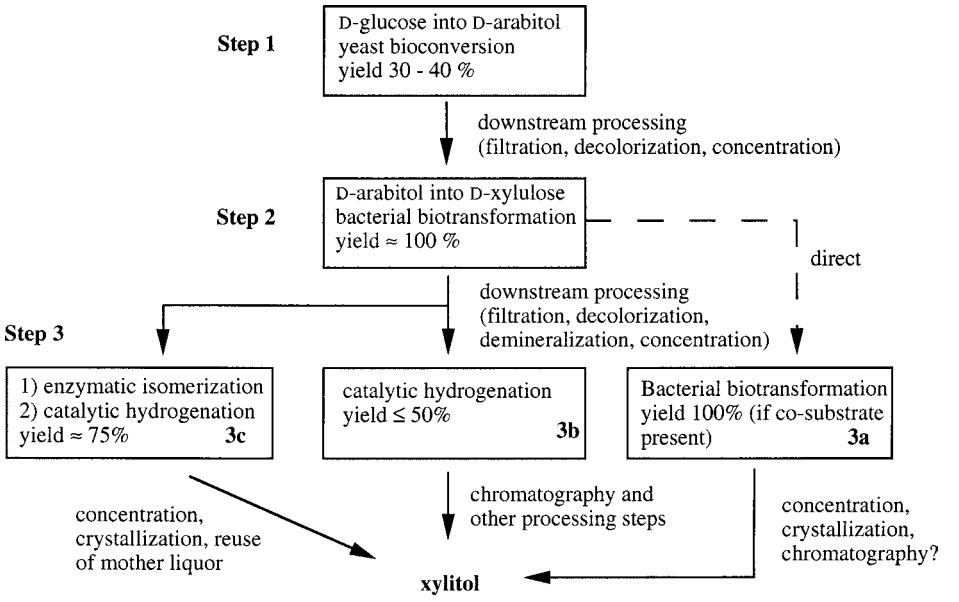

Fig. 1. Processes for production of xylitol from D-glucose.

yield of this step is only between 30 and 40% (step 1, Fig. 1). Therefore, the formation of D-arabitol is a major factor limiting the overall yield of the process for xylitol production from D-glucose. To the best of our knowledge, optimization of the microbial conversion of D-glucose into D-arabitol has not been investigated thoroughly so far, and, therefore, this step provides an important point of departure for improving the process. However, the establishment of optimal conditions for the formation of D-arabitol was not a goal of the present study.

In the second step of the process (Fig.1.), D-arabitol is oxidized into D-xylulose, and a wide range of bacteria, typically belonging to the genera *Gluconobacter* and *Arthrobacter (9,10)*, are capable of carrying out this reaction. Whole-cell biotransformation of D-arabitol leads to an initial accumulation of D-xylulose in typically 6–20 h and provides a yield of D-arabitol consumption of between 90 and 100% (step 2). The portion of xylitol present in D-xylulose may be as high as 30% at the time when exhaustive conversion of D-arabitol has been obtained *(9,10)*. Typically, it increases with increasing incubation time as a result of progressing D-xylulose reduction catalyzed by the cell *(9,10)* (step 3a). However, since the reasonably complete formation of xylitol by natural bacterial strains requires reaction times of 1 to 2 days and thus displays a very low productivity, catalytic hydrogenation of D-xylulose has been considered in patented xylitol processes *(7)* (step 3b). It yields a mixture of xylitol and D-arabitol in about equal proportions. To circumvent the incomplete stereoselectivity of the chemical catalysts and thus increase the yield of xylitol, a chemoenzymatic step has been developed in which glucose isomerase is used to promote isomerization of

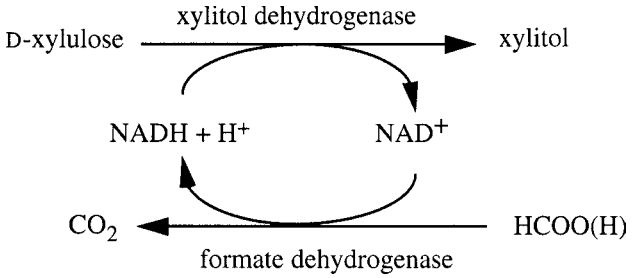

Fig. 2. Enantioselective reduction of D-xylulose by using xylitol dehydrogenase and NADH, and *in situ* coenzyme regeneration by formate dehydrogenase–catalyzed oxidation of formate anion.

D-xylulose into D-xylose *(7)* (step 3c). Unlike D-xylulose, D-xylose can only yield xylitol as reduction product.

Although xylitol produced via one of the routes shown in Fig. 1 can be crystallized as an almost pure chemical, the processes for xylitol production from D-glucose are not suitable for commercial practice. The most obvious limitations pertain to both yield (steps 1 and 3b) and productivity (steps 1 and 3a). Apart from the production of D-arabitol from D-glucose, which is a clear bottleneck in terms of yield, the final step of the three-step reaction sequence is another important factor of xylitol yield (steps 3b and c) and productivity (step 3a). Furthermore, it mainly determines the requirement for downstream processing because xylitol cannot be separated very easily from large amounts of contaminating D-arabitol.

In this article, we address the issue of D-xylulose reduction in the process for xylitol production from D-glucose. We propose an enzymatic approach, based on the use of NAD^+-dependent dehydrogenases, that avoids problems with stereoselectivity during carbonyl group reduction and enables xylitol formation at a good productivity. Recombinant xylitol dehydrogenase from the yeast *Galactocandida mastotermitis (11,12)*, produced in *Escherichia coli (13)*, was used as the key catalyst for xylitol production, and the well-known formate dehydrogenase from *Candida boidinii (14)* was used for the regeneration of NADH (Fig. 2). The new enzymatic step of D-xylulose reduction is compared to the microbial and chemicocatalytic reaction sequences described in the scientific *(6)* and patent literature *(7,9,10)*.

Materials and Methods

Chemicals

NAD^+ (97.7% pure) was from Seppim SA (Sees, France). D-Arabitol and ampicillin were purchased from Sigma (Deisenhofen, Germany). Isopropyl-β-D-thiogalactoside was obtained from Biotechnik GmbH (Gailberg, Germany). Yeast extract was from Merck (Darmstadt, Germany), and

casein peptone from Fluka (Buchs, Switzerland). All other chemicals were of reagent grade and obtained through local suppliers. Materials for protein chromatography were those described in recent publications *(11,12)*.

Microorganisms and Enzymes

Gluconobacter oxydans subsp. *suboxydans* DSM 2003 was obtained from DSM— Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). *G. suboxydans* was grown in liquid culture at 25°C using baffled Erlenmeyer flasks and a medium (pH 6.9) composed of the following: 75 g/L of D-mannitol, 16 g/L of peptone from casein, 8 g/L of yeast extract, and 1 g/L of $CaCO_3$. D-Mannitol was preferred over D-arabitol because growth of the organism on D-mannitol had been well established in previous experiments. Note that mannitol dehydrogenase catalyzes the oxidation of D-arabitol into D-xylulose *(15)* whereas D-arabitol dehydrogenase is thought to produce D-ribulose from D-arabitol *(8)*. Therefore, we did not expect an inducing effect specific to D-arabitol.

A typical cultivation of *G. suboxydans* started from agar plates and required a preculture of 25 mL as inoculum of the main culture of 250 mL. Agitation was at 130 rpm using an Infors model Multitron incubator (Bottmingen, Switzerland). The duration of the preculture and the main culture was approx 2 d each. Cells of the main culture were harvested by centrifugation (20 min at 16,000*g* and 4°C), washed twice with saline, and stored at 4°C until further use. Approximately 6 g/L of wet biomass was obtained.

Recombinant xylitol dehydrogenase from *G. mastotermitis* was produced in *E. coli* JM 109 using the plasmid expression vector pBTac1 described recently *(13)*. Cultivation of the bacterial strain was performed at 37°C and agitation of 110 rpm using LB medium supplemented with 60 mg/L of ampicillin. Production of xylitol dehydrogenase was induced by adding 0.45 m*M* isopropyl-β-D-thiogalactoside to the medium when the optical density of the culture at 600 nm had reached a value of about 1.8. Cells were harvested approx 8 h after the time of induction by centrifuging at 16,000*g* and 4°C for 20 min. Further workup of the cells and purification of xylitol dehydrogenase by biomimetic dye-ligand chromatography followed the procedures described for the isolation of the natural enzyme from *G. mastotermitis (11)*. An enzyme preparation with a specific activity of approx 80 U/mg (see next session) was obtained and concentrated by ultrafiltration (30 kDa cut-off) to approx 1 to 2 mg/mL. The enzyme was stored at 4°C.

Formate dehydrogenase from *C. boidinii* was obtained from ASA Spezialenzyme GmbH (Braunschweig, Germany). The commercial enzyme preparation had a specific activity of 2.6 U/mg of protein and contained approx 55 U/mL. In spite of its glycerol content (≥ 20%), the enzyme was not dialyzed before use, but it may be necessary to do so when high concentrations of formate dehydrogenase are employed in the reaction.

Enzyme Assays

The activity of xylitol dehydrogenase was measured at 25°C with 144 m*M* xylitol and 1 m*M* NAD^+as the substrates dissolved in 50 m*M* Tris-HCl buffer, pH 9.0. The activity of formate dehydrogenase was measured at 25°C with 600 m*M* sodium formate and 1.5 m*M* NAD^+ as the substrates dissolved in 50 m*M* Tris-HCl buffer, pH 7.0. The rates of formation of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) were recorded spectrophotometrically in both enzyme assays. Protein was determined using the Bio-Rad (Hercules, CA) dye-binding assay and bovine serum albumin (albumin fraction V; USB, St. Louis, MO) in concentrations of 0.1–1.0 mg/mL as the standard.

Preparation of D-Xylulose

The transformation of D-arabitol into D-xylulose was carried out in water using resting cells of *G. suboxydans*. Approximately 0.1 g of wet cell mass was suspended in 1.5 mL of water and added to 8.5 mL of D-arabitol solution. The final polyol concentration was between 0.1 and 0.5 *M* (15 and 75 g/L). The incubation was performed at 25°C in baffled Erlenmeyer flasks using agitation at 110 rpm. Samples were taken in regular intervals and the depletion of substrate and the formation of product were monitored over the reaction time by high-performance liquid chromatography (HPLC) analysis. After exhaustive turnover of D-arabitol had occurred, the bacterial cells were inactivated by heat treatment (≥70°C, 4 min) and separated from the bulk solution by ultracentrifugation (80,000 *g*, 20 min, 4°C). The clear supernatant was lyophilized.

Batchwise Synthesis of Xylitol

The substrates D-xylulose and sodium formate (each 0.1–0.3 *M*), and NAD^+ (0.1-1.0 m*M*), were dissolved in 100 m*M* Tris-HCl buffer, pH 7.5, containing 0.5 m*M* dithiothreitol (DTT) and 0.5 m*M* EDTA. The final volume after the addition of the enzymes (500–2000 U/L each) was 2 mL. The reactions were carried out in stoppered beakers with a total volume of 5 mL. The temperature was 25°C, and mixing was achieved by means of a magnetic stirrer at 60 rpm. Before the enzymes were added to start the reaction, the substrate solution was gassed with N_2 to remove dissolved O_2. Samples of 50 µL were taken in regular intervals to monitor the course of the reaction over time. Each sample was diluted seven-fold with water and treated as described in Analytical Procedures. The pH of the reaction mixtures was also measured regularly using an external microelectrode. Control of pH was not required and the pH was found to change from the initial value of 7.5 to 8.3.

When turnover of D-xylulose was complete, enzyme recovery was performed by means of ultrafiltration with a molecular mass cutoff of 30 kDa using Microsep centrifugal microconcentrator tubes (Filtron, Northborough, MA) for processing a volume of ≤2 mL. Centrifugation

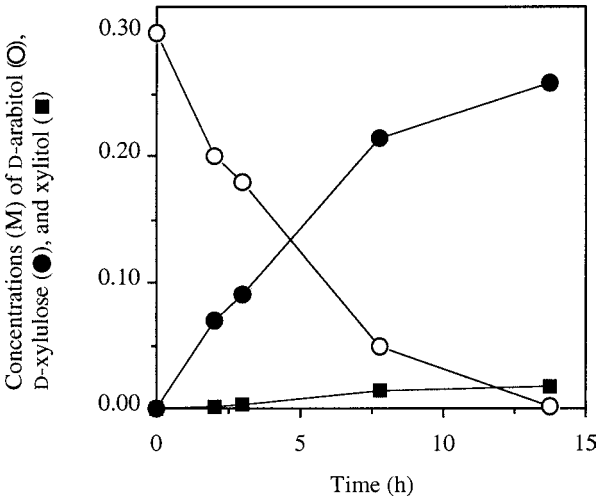

Fig. 3. Transformation of D-arabitol into D-xylulose using oxidation catalyzed by resting cells of *G. suboxydans*.

was carried out at 4°C and 5000*g* for the time required to concentrate the filtrate by a factor of approx 5. A tangential flow device with the same molecular-mass cutoff was used for enzyme recovery when volumes of up to 1 L had to be processed (MINI-ULTRASETTE; Filtron). A peristaltic tubing pump was employed to deliver a flow rate of between 100 and 300 mL/min to adjust the permeate flow rate (Watson-Marlow model 501U; Wilmington, MA).

Analytical Procedures

Carbohydrates were analyzed by HPLC using an Aminex HPX 87C column from Bio-Rad. The column was operated at 85°C, and elution was carried out at a flow rate of 0.7 mL/min by using 10 m*M* $Ca(NO_3)_2$ in water. Refractive index detection with a Merck ERC-7512 detector was used. For quantitative analysis, the peak areas were determined using the Merck integrator model D-2500. All samples were boiled for 3 min and centrifuged in order to precipitate and remove protein prior to analysis. The clear supernatants were used for HPLC analysis. The heat treatment was shown not to interfere with determination of the sugar concentrations.

Results and Discussion

Preparation of D-Xylulose

Since D-xylulose is not a commercial bulk chemical, it was synthesized in a preparative reaction on a laboratory scale. Figure 3 shows the dependence on incubation time of the biotransformation of D-arabitol into D-xylulose catalyzed by resting cells of *G. suboxydans* in water at 25°C.

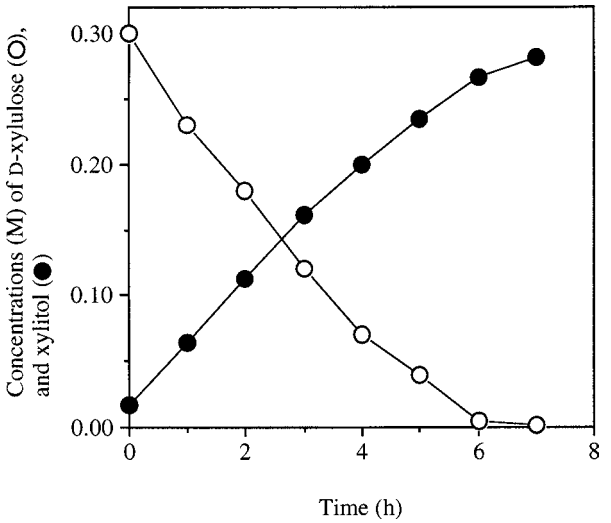

Fig. 4. Production of xylitol through the stereospecific, enzymatic reduction of D-xylulose. Each dehydrogenase was used at 1000 U/L. The concentration of NAD^+ was 0.5 m*M*.

The yield of D-xylulose was dependent on the substrate concentration and decreased with increasing concentration of D-arabitol: ≥95, 86, and 80% when using D-arabitol concentrations of 0.10 (15 g/L), 0.30 (46 g/L), and 0.50 *M* (76 g/L), respectively. The formation of a small amount of xylitol was observed, and this increased with increasing concentrations of D-arabitol. Xylitol corresponded to 5% of the total sugars present in the product mixture when an initial D-arabitol concentration of 0.3 *M* was used. The reaction time required to achieve exhaustive conversion of the substrate increased with increasing concentration of D-arabitol. Exhaustive conversion occurred at 5, 12, and 24 h for substrate concentrations of 0.1, 0.3, and 0.5 *M*, respectively. *G. suboxydans* may be capable of reducing the produced D-xylulose into xylitol, however, that reaction would require the addition of NADH or cosubstrates to generate reduction equivalents *(9,10)*. Because we considered the transformation of D-xylulose by soluble enzymes to be the more promising approach regarding achieving high productivity, studies with the microbial system toward direct xylitol production from D-arabitol were not pursued further. Use of soluble enzymes is expected to ensure the absence of mass transfer limitations of the reaction rates and allows the design and optimization of the process of coenzyme regeneration.

Batchwise Synthesis of Xylitol

Using the enzyme/coenzyme system described in Fig. 2, the turnover of D-xylulose was complete after 7 h and the xylitol yield approached 100% (Fig. 4). The NAD+ employed in the batch process was regenerated 600-fold. (Results are generally shown in molar concentrations to facilitate calculation of the coenzyme turnover number for each experiment.) Samples

taken at the end of the reaction were analyzed for remaining enzyme activities of xylitol dehydrogenase and formate dehydrogenase. The enzymes were found to be stable within limits of experimental error (±5%). Previous studies have shown that thiol protection by EDTA and sulfhydryl reagents such as DTT positively affects the stability of both dehydrogenases. Therefore, these two compounds were present at all times during the enzymatic reaction. Considering the costs of the reagents and the workup required to remove them from the product, it would be desirable to eliminate or at least minimize the amounts added. This provides an important goal for future studies to improve the enzymatic process.

We compared two different preparations of xylitol dehydrogenase for the conversion of D-xylulose: one that was partially purified by dye-ligand chromatography and another that corresponded to the enzyme in the crude cell extract of *E. coli*. The aim was to identify the simplest procedure for obtaining a technical-grade biocatalyst, possibly avoiding protein chromatography, which strongly dilutes the enzyme activity so that protein concentration by ultrafiltration is required for enzyme dosage in the process. We found no difference between the two preparations of xylitol dehydrogenase regarding production of xylitol and stability of enzyme activity in a batchwise reaction (results not shown).

Effect of Coenzyme Concentration

Since both xylitol dehydrogenase and formate dehydrogenase show a Michaelis-Menten-type dependence on the concentrations of NADH and NAD^+, one expects that the coupled enzyme system shown in Fig. 2 operates optimally at a high, saturating coenzyme concentration. However, product inhibition and the distribution of the total concentration of coenzyme between NADH and NAD^+ during the reaction are responsible for the fact that an optimum coenzyme concentration may exist for reactions catalyzed by two coupled dehydrogenases. In Fig. 5, the reaction time required to obtain a substrate turnover of 50% is plotted against the initial coenzyme concentration, using reaction conditions otherwise identical to those reported in Fig. 4. The results indicate an optimum concentration of NAD^+ which is between 0.5 and 0.75 m*M*. The NADH and NAD^+ concentrations that prevail during the synthesis of xylitol, and thus the optimum total coenzyme concentration are, however, dependent on the ratio of xylitol dehydrogenase and formate dehydrogenase, which was 1.0 for the experiments reported in Fig. 5. To optimize the enzymatic reaction for a wider range of experimental conditions than used here, modeling based on the kinetic parameters of both dehydrogenases should be employed *(16,17)*. The necessity of using modeling to understand the interactions of all reaction parameters and support further optimization of the process is stressed by our observation that an increase in the enzyme concentration from 500 to 2000 U/L using an activity ratio of 1.0 in each case did not lead to the expected four-fold increase in xylitol productivity. A 2.2-fold increase in

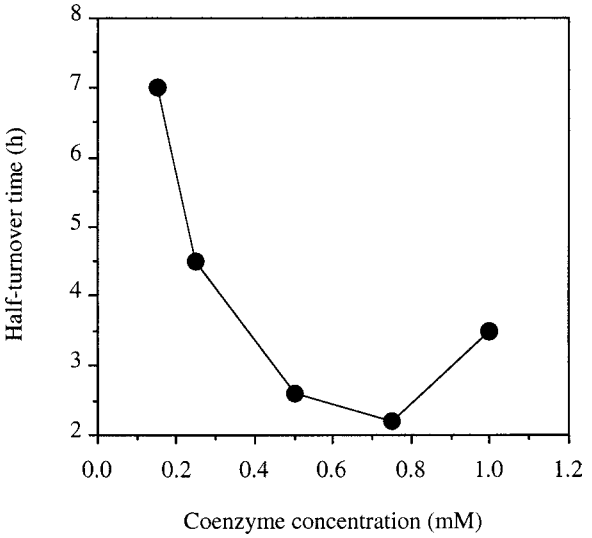

Fig. 5. Determination of coenzyme concentration for optimum productivity in production of xylitol from D-xylulose. The same conditions as in Fig. 4 were used.

productivity was measured at a turnover level for the substrate of 30%, i.e., when substrate was not limiting.

Effect of Substrate Concentration

The time required to obtain a 50% level of substrate conversion increased in an almost linear fashion with increasing concentrations of D-xylulose from 0.1 to 0.3 *M* (results not shown). Therefore, the observed xylitol productivity of 46 m*M*/h or 7 g/(L·h) was similar at the different substrate concentrations employed (0.10; 0.20; and 0.30 *M*). This result implies that both enzymes were saturated with substrate under all conditions used, which is in accordance with relatively small K_m values for xylitol dehydrogenase and formate dehydrogenase of 10 m*M* D-xylulose and 13 m*M* sodium formate, respectively.

Reuse of Enzymes Using Ultrafiltration

Using ultrafiltration with a molecular mass cutoff of 30 kDa, xylitol dehydrogenase (160 kDa) and formate dehydrogenase (80 kDa) could be recovered in yields of typically 85–95% after the enzymatic conversions of D-xylulose and formate had proceeded to completion. Minor losses of enzyme activity occurred during the ultrafiltration and not the reaction. By using a fill-and-draw reaction sequence in which product was removed as ultrafiltration permeate, the original xylitol dehydrogenase and formate dehydrogenase could be used for at least six rounds of substrate conversion of which each lasted 8 h (Fig. 6). To confirm observations made on a 2-mL scale, the enzymatic transformation of D-xylulose was carried out in a volume of 100 mL under otherwise identical conditions as those in Fig. 6.

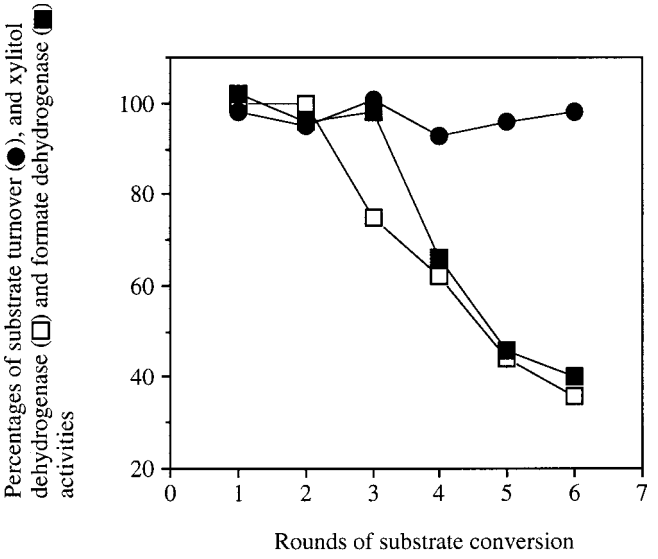

Fig. 6. Enzyme recycle in coenzyme-dependent production of xylitol. Other conditions: concentrations of D-xylulose and formate of 0.3 *M* (added in each round); NAD^+ concentration of 0.5 m*M* (added in each round), 1000 U/L of each enzyme. Each round of conversion lasted 8 h.

Recovery of the enzymes was performed by using crossflow ultrafiltration that was operated at a permeate flow rate of 160 mL/h. Approximately 90% of the original activity of both enzymes was found in the filtrate and ready to be used in a new cycle of xylitol production.

Advantages of Replacing Chemicocatalytic Hydrogenation Step Using Enzymatic Catalysis

We discuss next the possible benefits of integrating the enzymatic reduction step (Fig. 2) into a process for the production of xylitol from D-glucose. To stress the advantages gained by using enzyme technology, we point out first the drawbacks inherent to the different process schemes depicted in Fig. 1 (steps 3a–c). The discussion, however, does not focus on a comparison of D-glucose- and D-xylose-based processes for xylitol production.

When using catalytic hydrogenation of D-xylulose, the yield of xylitol is not satisfactory: the reduced product contains nearly 50% of D-arabitol and thus partly regenerates the substrate of step 2 in the process (Fig. 1). There has been significant progress recently in the development of metal catalysts that allow enantiodifferentiating hydrogenation of prochiral ketones *(18)*, including ketoses such as D-fructose *(19)* and perhaps D-xylulose. One could expect that the xylitol yield might be improved substantially by using tailored Raney metal catalysts.

Enzymatic isomerization of ketoses yielding the corresponding aldose (e.g., D-xylulose isomerization into D-xylose), which is reduced to a

stereochemically defined product, has been a useful method to increase the yield of the desired polyol in the reductions of D-xylulose to xylitol *(7)* and D-fructose to D-mannitol *(20)*. The procedure of isomerization is simple and usually complete within a few h, and glucose (xylose) isomerase is an enzyme produced commercially for large-scale applications. If we consider only the results for a batchwise hydrogenation of a mixture of D-xylulose and D-xylose at equilibrium, a xylitol yield of approx 75% from the initial D-xylulose could be achieved in that way *(7)*. However, in spite of the significant improvement in xylitol yield by using enzymatic isomerization of D-xylulose, the requirement for the addition of a new process step is a considerable disadvantage because it decreases productivity and contributes to process costs.

An attractive alternative is, therefore, the "direct" conversion of D-arabitol into xylitol by using bacteria *(9,10)*. However, the product yield and the reaction rates, measured as xylitol formed, delivered by resting cells of *Arthrobacter* or *Gluconobacter* strains appear to be too low for technologic applications. When sugars such as D-glucose or polyols such as glycerol were added to support the generation of the required reduction equivalents, the observed xylitol yield did increase up to three-fold to a value of near 100%. The important question not answered in a satisfactory fashion so far is, is whether the achievement made by cosubstrate addition is lost during downstream processing when,e.g., excess glycerol must be removed?

The enzymatic reaction developed in our work provided a theoretical yield of xylitol from D-xylulose. A reasonably high xylitol concentration of up to 0.24 *M* (36 g/L) was obtained. However, there is no obvious reason why a substrate solution containing a concentration of D-xylulose even higher than 0.24 *M* should not be converted fully into xylitol. The maximum D-xylulose concentration obtained through microbial oxidation of D-arabitol may be limited to approx 0.3 *M* (45 g/L) since it was shown that the yield of D-xylulose decreased significantly as the concentration of D-arabitol was increased. Concentration of the D-xylulose solution before enzymatic reduction could thus be an option.

A high xylitol productivity was obtained even at moderate enzyme doses of 1000 U/L. The affinities of the enzymes for their substrates are relatively high, and product inhibition by xylitol is not significant. Therefore, this implies that turnover in a stirred-tank reactor can proceed at a fast rate up until high substrate conversion levels have been reached. However, two dehydrogenases and NAD^+ are required, and it is a common prejudice in industry that the costs of the three reactants make the entire process intrinsically uneconomical. Therefore, one should note that the activities of xylitol dehydrogenase and formate dehydrogenase were fully stable during the biotransformation and could be recovered from the product solution with a yield of 90% by using ultrafiltration. To regenerate the coenzyme, which would otherwise be lost after each cycle of substrate conversion, the ultrafiltration should be replaced by nanofiltration *(16,21)*. By so doing, both the enzymes and the coenzymes NADH and NAD^+ would remain in

the filtration retentate and xylitol removed with the filtrate. Key goals for future work aimed at improving the final step of the process would thus be the demonstration of the utility of nanofiltration and based thereon the evaluation of enzyme and coenzyme costs. In addition, a simplification of the reaction medium regarding buffer components and enzyme stabilizers will be important. Considering the overall process for xylitol production from D-glucose, the optimization of the conversion of D-glucose into D-arabitol will probably be the most important issue to be solved in order to increase the ability of the D-glucose-based process to compete with classic D-xylose-based processes. The available data on yield currently still favor the D-xylose-based approaches.

References

1. Parajo, J. C., Dominguez, H., and Dominguez, J. M. (1998), *Bioresour. Technol.* **65,** 191–201.
2. Parajo, J. C., Dominguez, H., and Dominguez, J. M. (1998), *Bioresour. Technol.* **65,** 203–212.
3. Parajo, J. C., Dominguez, H., and Dominguez, J. M. (1998), *Bioresour. Technol.* **66,** 25–40.
4. Meinander, N. Q. and Hahn-Hägerdal, B. (1997), *Biotechnol. Bioeng.* **54,** 391–399.
5. Roca, E., Meinander, N., and Hahn-Hägerdal, B. (1996), *Biotechnol. Bioeng.* **51,** 317–326.
6. Onishi, H. and Suzuki, T. (1969), *Appl. Microbiol.* **18,** 1031–1035.
7. Leleu, J. -B., Duflot, P., and Caboche, J. -J. (1993), US patent no. US005096820.
8. Wong, B., Murray, J. S., Castellanos, M., and Croen, K. D. (1993), *J. Bacteriol.* **175,** 6314–6320.
9. Suzuki, S., Sugiyama, M., Mori, M., Mihara, Y., and Yokozeki, K. (2000), European patent no. EP1026255A1.
10. Sugiyama, M., Suzuki, S., Mihara, Y., Hashiguchi, K., and Yokozeki, K. (2000), European patent no. EP1026254A1.
11. Lunzer, R., Mamnun, Y., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1998), *Biochem. J.* **336,** 91–99.
12. Lunzer, R., Ortner, I., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1998), *Biocat. Biotrans.* **16,** 333–349.
13. Habenicht, A., Motejadded, H., Kiess, M., Wegerer, A., and Mattes, R. (1999), *Biol. Chem.* **380,** 1405–1411.
14. Schütte, H., Flossdorf, J., Sahm, H., and Kula, M. -R. (1976), *Eur. J. Biochem.* **62,** 155–160.
15. Slatner, M., Nidetzky, B., and Kulbe, K. D. (1999), *Biochemistry* **38,** 10,489–10,498.
16. Nidetzky, B., Neuhauser, W., Haltrich, D., and Kulbe, K. D. (1996), *Biotechnol. Bioeng.* **52,** 387–396.
17. Kragl, U., Vasic-Racki, D., and Wandrey, C. (1996), *Bioprocess Eng.* **14,** 291–297.
18. Osawa, T., Harada, T., and Osamu, T. (2000), *Top. Catal.* **13,** 155–168.
19. Mohr, T., Schwarz, E., and Mackert, P.-J. (2001), World patent no. W01/00550A1.
20. Makkee, M., Kieboom, A. P. G., and van Bekkum, H. (1985), *Starch* **37,** 136–141.
21. Seelbach, K. and Kragl, U. (1997), *Enzyme Microb. Technol.* **20,** 212–235.