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Purification of Xylitol Dehydrogenase and Improved Production of Xylitol by Increasing XDH Activity and NADH Supply in *Gluconobacter oxydans*

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ABSTRACT: *Gluconobacter oxydans* is known to be a suitable candidate for producing xylitol from D-arabitol. In this study, the enzyme responsible for reducing D-xylulose to xylitol was purified from *G. oxydans* NH-10 and characterized as xylitol dehydrogenase. It has been reported that XDH depends exclusively on NAD^+/NADH as cofactors with a relatively low activity, which was proposed to be the direct reason for its limiting the overall conversion process. To better produce xylitol, an engineered *G. oxydans* PXPG was constructed to coexpress the XDH gene and a cofactor regeneration enzyme (glucose dehydrogenase) gene from *Bacillus subtilis*. Activities for both enzymes were more than twofold higher in the *G. oxydans* PXPG than in the wild strain. Approximately 12.23 g/L xylitol was obtained from 30 g/L D-arabitol by resting cells of the engineered strain with a conversion yield of 40.8%, whereas only 7.56 g/L xylitol was produced by the wild strain with a yield of 25.2%. These results demonstrated that increasing the XDH activity and the cofactor NADH supply could improve the xylitol productivity notably.

KEYWORDS: *xylitol, xylitol dehydrogenase, Gluconobacter oxydans, coexpression, NADH regeneration, bioproduction*

INTRODUCTION

Xylitol is a naturally occurring pentahydroxy sugar alcohol. It has a degree of sweetness similar to that of sucrose, hence it is often used as an alternative sweetener which assists weight loss and inhibition of dental caries without compromising the taste. Considering that its metabolism is insulin independent, xylitol is generally deemed suitable for diabetics. Recognition of such advantages brings about wide applications of xylitol in the food and chemical industries.[1−5] According to an authoritative report, the recent annual xylitol market was estimated to be over $340 million, priced at $4−5/kg.[22]

Xylitol can be prepared chemically through reduction of D-xylose. However, the energy consumption, pollutant output, and costs involved in the downstream isolation process are considerably high.[6] In the past decades, a two-step process involving D-glucose as the initial substrate has been developed as an alternative because of the obvious technological and economic advantages of D-glucose over D-xylose. The process can be considered as a combination of two sets of reactions described in previous reports.[7−10] D-glucose is converted into D-arabitol by *osmophilic* yeasts in the first step, followed by the transformation into xylitol using *Gluconobacter oxydans* which hosts membrane-bound D-arabitol dehydrogenase (m-ArDH) and NAD-dependent xylitol dehydrogenase (XDH). An almost complete conversion is usually achieved with m-ArDH whereas the conversion effciency for the subsequent dehydrogenation stays about 25%.[7−9] It is therefore deduced that XDH catalyzes the rate-limiting step and controls the overall biotransformation process, probably because of its limited activity and the coenzyme deficiency. From a practical point of view, an increase in the conversion rate of D-xylulose to xylitol can definitely improve the xylitol yield and refine the manufacturing process.

To date, various attempts have been made to elevate the xylitol yield. M. Sugiyam enhanced xylitol productivity by overexpressing the *xdh* gene in *G. oxydans*, which led to increased XDH activity up to 11-fold higher than that of the wild-type strain. Nevertheless, there is still room for further improvement: The xylitol yield was less than 30%. A sufficient supply of NADH on a reducing equivalent basis was demanded to overcome this limiting step, given that XDH depends on NADH exclusively.[11] Later on, G. Mayer employed the purified XDH and formate dehydrogenase to catalyze xylitol generation from D-xylulose. When 0.5 mM NAD^+ was added for NADH regeneration, a yield close to 100% was achieved.[12] However, the high cost and limited availability of pyridine cofactors hindered the economic viability of industrial-scale biotransformation using XDH in situ cofactor regeneration step.[32,33] The most convenient and useful method for regenerating the needed cofactors is to employ dehydrogenases that recycle nicotinamide coenzymes.[34]

As a solution to the above-mentioned problems, a coexpression system was established to improve the XDH activity and coenzyme regeneration. In a recent study by P. Zhou,[10] D-xylulose was prepared from D-arabitol by *G. oxydans* NH-10, and some coexpression plasmids in *Escherichia coli* Rosetta (DE3) were constructed for efficient xylitol production.

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As a result, the productivity of xylitol was significantly enhanced to 92% yield, but the safety, economy, and operation complexity issues still remain in their systems.

In this study, the enzyme responsible for the reduction of D-xylulose to xylitol was purified and characterized. Moreover, the coexpression plasmid for enhancement of the XDH activity together with its cofactor supply was constructed and transformed into the wild *G. oxydans* strain. According to the results and analysis, we hope to establish an efficient and concise route for xylitol production by *G. oxydans*.

MATERIALS AND METHODS

Strains, Materials, Culture Media, and Conditions. The xylitol producing strain *G. oxydans* NH-10 was isolated from soil and deposited at China General Microbiological Culture Collection Center with accession number CGMCC 2709. The host strains *E. coli* JM109 and BL21 (DE3) were obtained from Novagen and routinely cultured in Luria–Bertani (LB) medium at 37 °C.

The seed culture medium (SCM) for *G. oxydans* was composed of the following (per liter): 30 g of glucose, 5 g of yeast extract, 3 g of peptone, 10 g of D-arabitol, and 5 g of $CaCO_3$. The sugar-rich medium (SRM) used for high-density cultivation was composed of the following (per liter): 73 g of sorbitol, 20 g of yeast extract, 1.5 g of $(NH_4)_2SO_4$ and KH_2PO_4, and 0.47 g of $MgSO_4{\cdot}7H_2O$. *G. oxydans* NH-10 was initially cultured on SCM for 24 h at 30 °C and then inoculated into 4.0 L of fermentation medium in a 7.5 L tank and cultured at 30 °C under optimal aeration and pH conditions. Restriction enzymes, Bacterial Genomic DNA Extraction Kit, T4 DNA ligase, exTaq polymerase, as well as DNA and protein markers were obtained from TaKaRa (Dalian, China).

Purification and Characterization of the Target Enzyme. *Preparation of Cell-Free Extract.* All purification procedures were performed at 4 °C. The reagents DTT, PMSF, and glycerol were added at appropriate concentrations to the buffer when necessary to maintain the enzyme activity. The cells were harvested by centrifugation and washed twice with 100 mM potassium phosphate buffer (KPB; pH 6.5). The precipitate was suspended in the same buffer and then disrupted by the sonication. After centrifugation, the supernatants were obtained as crude enzyme extractions, which were passed through a 0.45 μm filter before the purification procedure.

Purification of the Responsible Enzyme from G. oxydans NH-10. Ammonium Sulfate Precipitation. The supernatant was fractionated by ammonium sulfate between 20% and 60% saturation. Then the precipitate formed was dissolved and dialyzed against in KPB buffer.

Anion Exchange Chromatography. The dialyzed enzyme solution was loaded onto a DEAE-Sepharose fast-flow column (φ16 mm × 200 mm) previously equilibrated with binding buffer A 50 mM KPB (pH 7.5). The elution was done by a linear gradient of 0–40% buffer B composed of binding buffer A and 1 M sodium chloride in addition. Fractions containing XDH activity were pooled, dialyzed, and concentrated.

The enzyme was further depurated with a second ion-exchange resin named Source 15Q. The resulting enzyme from the preceding step was put onto the column (Tricorn, φ10 mm × 100 mm) equilibrated with the binding buffer C 20 mM Histidine (pH6.3). The column was washed, and the enzyme was eluted with a linear gradient at a flow rate of 1 mL/min, giving a symmetrical activity curve which agreed with the elution of protein monitored by the optical absorbance at 280 nm.

HiPrep Sephacryl S-200 HR Column Chromatography. The treated active fractions were subsequently applied into a HiPrep Sephacryl S-200 HR column (φ16 mm × 600 mm) equilibrated with buffer E. The XDH enzyme was eluted with the same buffer at 0.5 mL/min used as purified enzyme in the following research.

Properties of Xylitol Dehydrogenase from G. oxydans. To determine the optimum temperature and pH of XDH, a standard enzyme assay was performed at different temperatures and pH values. The temperature and pH stability, as well as the kinetic constants K_M and V_{max}, were investigated with the method described by Nina.[13]

Quadrupole Time-of-Flight Mass Spectrometer Analysis of the Purified Protein. Purified XDH was isolated from the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) preparation and digested with 10 μL of trypsin at 4 °C for 30 min. The product was subjected to mass mapping by time-of-flight tandem mass spectrometry. The data obtained were analyzed using the GPS (Applied Biosystems)-MASCOT (Matrix Science, London, U.K.) search software.[14]

Cloning of xdh Gene from G. oxydans and Expression in E. coli. The *xdh* gene was amplified from the *G. oxydans* genome by polymerase chain reaction (PCR) using sense- and antiprimers (Table 1). The purified PCR product was digested with the restriction enzymes and ligated with expression vector designated as pET28a::*xdh*, which was used to transform the *E. coli* strain BL21 (DE3). The transformants were shaken at 37 °C in LB medium containing ampicillin first. When the OD_{600} of the cell suspension reached 0.6, the cells were induced in LB with subsequent addition of 0.5 mM IPTG on 250 rpm shaking incubator with temperature 30 °C.

Table 1. Oligonucleotide Primers for PCR in This Study

primer name	sequences (5′-3′)	digestion site
xdh-sence[a]	GGAATTCATGTCGAAGAAGTTTAACGG	*EcoR* I
xdh-anti[a]	CCCAAGCTTTTAACCAGCAA	*Hind* III
xdh-F	ATCTCGAGCAGAGTTTGAGGCATTCG	*Xho* I
xdh-R	AGAAGCTTATCAACCGCCAGCAATCG	*Hind* III
gdh-F	CGGAATTCACAAATGGAGGAGGATGT	*EcoR* I
gdh-R	ATGAGCTCTTATGTTTAACCGCGGC	*Sac* I
P*tufB1*-F	ATGGTACCTATCAGGTTCCGGTTGAAG	*Kpn* I
P*tufB1*-R	TAACTCGAGACCTGGAACGGGAGTAAG	*Xho* I
P*tufB2*-F	CTAAGCTTTATCAGGTTCCGGTTGAAGTC	*Hind* III
P*tufB2*-R	AAGAATTCACCTGGAACGGGAGTAAGAC	*EcoR* I

[a]Primers for section "Cloning of xdh gene from G. oxydans and expression in E. coli".

Construction of the Overexpression and Coexpression Plasmids. The coexpression plasmid for *xdh* and *gdh* was constructed to improve XDH activity and coenzyme regeneration in *G. oxydans* NH-10. The fragments of *xdh*, *gdh*, and promoter P_{tufB} were first amplified with the primers listed in Table 1. Then, the *xdh* or *gdh* was attached to the promoter P_{tufB}, and so the fragments P_{tufB}-*xdh*, P_{tufB}-*gdh* were obtained. Next, both the expression vector pBBR1MCS-5 and fragment P_{tufB}-*xdh* were double-digested with *Kpn* I and *Hind* III first and ligated with the help of T4 ligase, yielding the overexpression plasmids pBBR-P_{tufB}-*xdh*. Finally, the coexpression plasmid containing P_{tufB}-*xdh* and P_{tufB}-*gdh* was constructed similarly (Figure 1).

Expression of the Recombinant Vectors in *G. oxydans*. The transformation of expression plasmids into *G. oxydans* NH-10 was carried out through the triparental conjugation as previously reported.[15] *E. coli* JM109 bearing the expression vector and *E. coli*

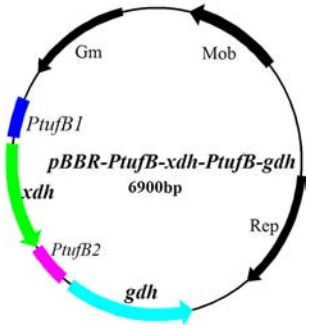

Figure 1. Diagrammatic map of the coexpression plasmid pBBR-P_{tufB}-*xdh*-P_{tufB}-*gdh*.

HB101 harboring the mobilizing plasmid pRK2013 were used as the donor and helper strain, respectively. These three strains were cultured to late exponential phase, centrifuged, washed, and resuspended in sorbitol-rich medium and then mixed at a ratio of 3:3:1. This mixture was spread onto sorbitol agar plates without antibiotics and incubated overnight at 30 °C. The cultures were then scraped from the plates and spread onto selective sorbitol agar plates containing 50 μg/mL ampicillin. The plates were incubated at 30 °C for 2−4 days until resistant colonies appeared. Recombinant strains were identified by direct sequencing and restriction enzyme reaction. The strains named *G. oxydans* PX for overexpression *xdh* and *G. oxydans* PXPG for coexpression *xdh* together with *gdh* were finally obtained.

Biotransformation of D-Arabitol to Xylitol by *G. oxydans* Resting Cells Systems. Cultivation and collection of individual strains were performed according to the description in aforementioned sections. The biotransformation of D-arabitol to xylitol was achieved with shake flasks. Resting cells were dispersed at a concentration of 10% (w/v) in a reaction solution containing 100 mM sodium phosphate buffer (pH 6) and 3% (w/v) D-arabitol and incubated at 30 °C on a rotary shaker for 50 h in all. The first reaction was the oxidation of D-arabitol to D-xylulose at a rotation rate of 220 rpm for 8−10 h. The second reaction was reduction for the transformation of D-xylulose to xylitol, which was performed after 5% glucose (w/v) was added to the mixture and the rotation rate was reset to 50 rpm.[10]

Analytical Methods. *Dehydrogenase Activity Assay.* The XDH and GDH activities were determined according to a previously reported method.[10]

Detection of Substance Concentration. The concentration of proteins was determined by the Bradford method [16] with bovine serum albumin as the standard. The holo-enzyme molecular weight of XDH was determined by gel filtration with a Sephacryl S-200 column (10 mm × 600 mm). Denaturing SDS-PAGE was performed as described by Laemmli [17] to determine the *apo*-enzyme molecular weight and purity. The concentrations of D-arabitol, D-xylulose, and xylitol in this study were determined by a previously reported method.[10]

RESULTS

Purification and Identification of Xylitol Dehydrogenase. Table 2 summarizes the enzyme purification procedure.

Table 2. Purification of Xylitol Dehydrogenase from *G. oxydans* NH-10

steps	protein/mg	activity/U	specific activity/(U/mg)	yield/%	purification fold
cell-free extract	1312.0	275.0	0.21	100	1
ammonium sulfate precipitation	561.10	233.4	0.42	84.1	2
DEAE FF column	41.50	171.0	4.01	62.2	19
Source 15Q column	1.60	129.6	80.85	47.1	385
Sephacryl S-200	1.10	108.4	98.50	39.4	469

The enzyme with XDH activity was purified to an apparent homogeneity at a recovery yield of 39.4%. Specific activity increased ∼469-fold during the purification from 0.21 to 98.5 U/mg. The size of the enzyme was determined to be 113 000 through gel filtration on a Sephacryl S-200 column. However, on the SDS−PAGE gel, purified enzyme solution appeared as a single protein band at a molecular weight of approximately 28 000. Therefore, it can be deduced that target enzyme has a homotetrameric structure similar to that of the xylitol dehydrogenase.

The amino acid sequence information of the protein band was determined by Q-TOF-MS, and the results were analyzed using the Mascot algorithm.[14,18] In the peptide mass fingerprint analysis, the masses obtained from the excised protein band showed the highest correlation with xylitol dehydrogenase from *G. oxydans* 621H, with a peptides sequence coverage of 84%. The nucleotide fragment coding for the purified enzyme was amplified from *G. oxydans* NH-10 genome and sequenced. There are 789 basic groups in the open reading frame, encoding 262 amino acids, which is 99% identical to xylitol dehydrogenase from *G. oxydans* 621H. An expression vector containing the ORF under the control of T7 promoter was constructed and expressed via standard procedure. The His-tagged XDH was one-step purified by the nickel metal-affinity column with a specific activity of 14.6 U/mg and tested on SDS−PAGE , resulting in a clear single protein band corresponding to 30 ± 1 KDa on SDS−PAGE.

Assay of Physiological and Biochemical Properties. *Effect of pH and Temperature on XDH Activity and Stability.* XDH activity was measured at various pH levels ranging from 4.0 to 12.0. Figure 2A shows that XDH was most active at pH

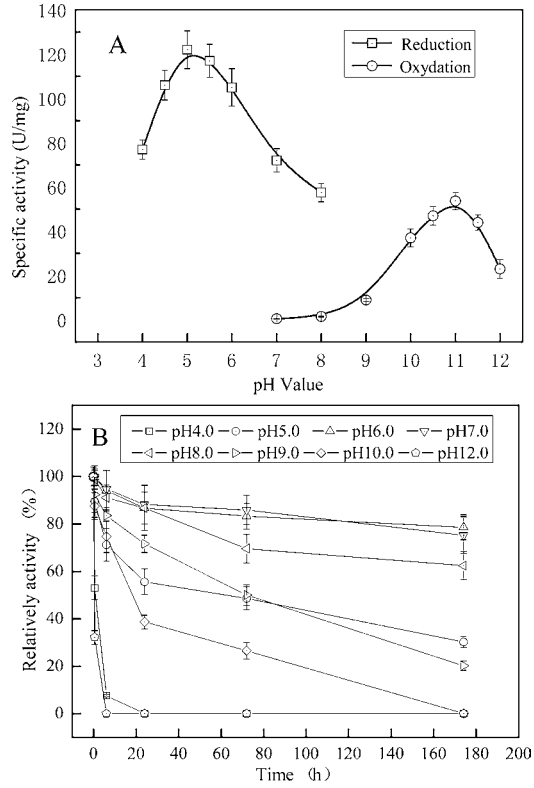

Figure 2. (A, B) Effects of pH on the activity and stability of XDH. In this study, four different buffers were used: sodium acetate (4.0−6.0), sodium phosophate (6.0−8.0), Tris-HCl (8.0−10.0), and NaOH−glycine (9.0−12.0) buffer.

10.5−11.0 for oxidation and pH 5.5 for reduction. XDH is relatively stable from pH 6.0 to 8.0, but the activity decreased notably in basic environment. The activity diminished significantly at pH 4.0 and 10.0 and vanished at pH 12.0 for 60 min (Figure 2B). Maximal oxidation activity at an alkaline pH and maximal reduction activity at an acidic pH are the common features of many polyol dehydrogenases from diverse

microbial systems.[19−21] The optimum temperatures of XDH for the oxidation and reduction reactions were 35 and 30 °C, respectively (Figure 3a). Studies on temperature stability were

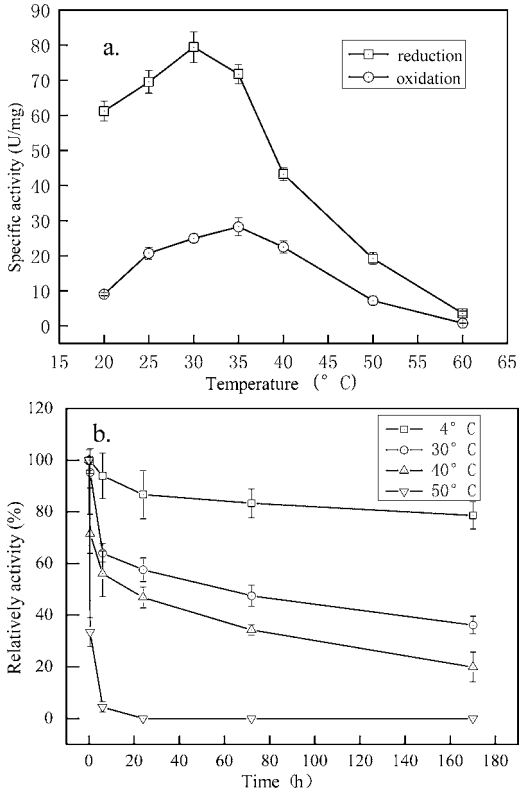

Figure 3. (a, b) Effects of temperature on the activity and stability of XDH. In this experiment, pH 6.0, 0.1 M sodium phosphate was used as the reaction buffer.

conducted. The rapid inactivation of the enzyme was observed over 40 °C, while nearly 50% activity at 30 °C and 80% activity at 4 °C was retained when stored in 100 mM KPB (pH 6.0) for 7 days (Figure 3b).

The Kinetic Parameters and Substrate Specificities of XDH. During the enzyme assays, XDH showed oxidation activities toward several alcohols in the presence of NAD^+, as well as reduction activities toward ketoses with NADH as the cofactor. The corresponding kinetic parameters were tabulated below (Table 3). Between the two cofactors, XDH apparently

Table 3. Kinetic Parameters of Purified Xylitol Dehydrogenase

substrate	V_{max}(mmol/mL·min)	K_M(mmol/L)	V_{max}/K_M
xylitol	103.1	147	0.7
D-xylulose	142.2	68.7	2.1
NAD^+	666.7	85.9	7.8
NADH	1209.1	57.8	20.9

preferred NADH to NAD^+, which indicated that XDH catalyzed a pair of reversible reactions of which reduction is usually in the ascendant. On the other hand, no measurable activities were detected when $NADPH/NADP^+$ was selected as the coenzyme, indicating that XDH was fed on $NADH/NAD^+$ exclusively.

Activity of XDH was tested against several substrates (Table 4). The higher the specific activity, the more competitive the

Table 4. Specific Activities of the Purified Xylitol Dehydrogenase[a]

substrate	product	specific activity (U/mg)	relative activity (%)
xylitol	D-xylulose	102.52 ± 5.53	100.00
D-sorbitol	L-sorbose	156.12 ± 6.71	152.29
meso-erythritol	NT	0.76 ± 0.05	0.74
D-mannitol	D-fructose	12.89 ± 3.09	12.57
D-arabitol	NT	6.74 ± 1.27	6.57
D-xylulose	xylitol	563.98 ± 5.62	100.00
D-fructose	D-mannitol	180.40 ± 4.20	31.99

[a]The recombinant XDH was assayed for reactions with various substrates under standard assay conditions described in the experimental procedures. Each value was the mean of triplicate measurements. NT: not tested.

substance is as a substrate for the enzyme in discussion. Hence, in terms of oxidation, D-sorbitol was the most competitive followed by xylitol, D-mannitol, and D-arabitol. As for the reduction, D-xylulose has an activity more than threefold higher than that of D-fructose. On the other hand, the specific activity of D-xylulose reduction was several folds higher than that of xylitol oxidation. Considering the kinetic parameters of XDH together, D-xylulose was prior to xylitol as the optimal substrate for XDH.

Construction and Expression of the Recombinant Vectors in *G. oxydans*. The plasmids for XDH overexpression and coenzyme regeneration were constructed and transformed into *G. oxydans* NH-10 by a triparental conjugation method. The strain harboring the pBBR-P_{tufB}-*xdh* plasmid was named as *G. oxydans* PX, whereas the other strain harboring the pBBR-P_{tufB}-*xdh*-P_{tufB}-*gdh* was named as *G. oxydans* PXPG. The plasmids in the recombinant strains were extracted and subsequently confirmed by PCR and restriction enzyme digestion. Biochemical performance of these strains was assessed and intercompared as well. The growth curves were meticulously investigated with the same conditions (data not shown). It has been apparent that the recombinant strains grew at approximately the same rate as the wild-type strain, giving the impression that both the expression vectors do not affect the physiological status of *G. oxydans*. Table 5 shows the

Table 5. XDH and GDH Activity of *G. oxydans* NH-10, *G. oxydans* PX, and *G. oxydans* PXPG[a]

activity (U/mg)	*G. oxydans* NH-10	*G. oxydans* PX	*G. oxydans* PXPG
XDH	0.05 ± 0.01	0.12 ± 0.04	0.10 ± 0.02
GDH	0.03 ± 0.01	0.04 ± 0.01	0.08 ± 0.01

[a]Each value is an average (±: standard error of the mean) of three parallel experiments.

measured XDH and GDH activities in these strains. The XDH activity of *G. oxydans* PXPG was about twofold higher than that of *G. oxydans* NH-10, while the activity of GDH was increased by about threefold.

Production of Xylitol from D-Arabitol by *G. oxydans* Resting Cells. With the reported resting cells reaction system, a detailed study on the time course of xylitol production was carried out in shake flasks. Figure 4 depicts the differences of xylitol formation among the strains. The xylitol production of the strain *G. oxydans* NH-10 was only 4.88 g/L (~20%), if D-glucose was not added into the system (Table 6). Then this was

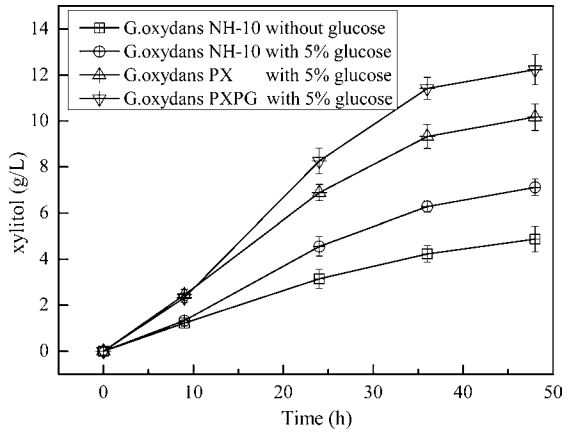

Figure 4. Study on the production of xylitol from D-arabitol by the wild and recombinant strains. Each value is an average (±: standard error of the mean) of three parallel experiments.

Table 6. Production and Yield of Xylitol from D-Arabitol Using Different Strains[a]

strains	substrate	co-substrate	xylitol (g/L)	yield (%)
G. oxydans NH-10	30g/L D-arabitol	no D-glucose	4.88 ± 0.55	16.3
G. oxydans NH-10	30g/L D-arabitol	5% (w/v) D-glucose	7.56 ± 0.36	25.2
G. oxydans PX	30g/L D-arabitol	5% (w/v) D-glucose	10.17 ± 0.57	33.9
G. oxydans PXPG	30g/L D-arabitol	5% (w/v) D-glucose	12.23 ± 0.65	40.8

[a]Each value is an average (±: standard error of the mean) of three parallel experiments.

elevated to 7.56 g/L after 5% (w/v) D-glucose was added to provide abundant NADH for XDH. Furthermore, the level of xylitol production reached 12.23 g/L when *G. oxydans* PXPG was used in the bioconversion, which was 20.3% and 61.8% more than that of *G. oxydans* PX (10.17 g/L) and *G. oxydans* NH-10, respectively. It is therefore concluded that high-level *xdh* expression and cofactor regeneration would result in distinctively improved xylitol productivity in recombinant *G. oxydans* PXPG.

DISCUSSION

As a general pentitol, xylitol has received intensive attention recently, particularly in areas of microbial production and metabolic engineering.[22] It is known to all that xylitol can be obtained from xylose reduction by *Candida* sp., *Debaryomyces hansenii*, and engineered *Escherichia coli*, etc.[23−25] However, the microbial production from D-glucose by biocoupling conversion[10] proved to be somehow less harmful to the environment, which can be realized by a two-step process. Furthermore, *G. oxydans* is of great concern in the conversion due to not only its role as the producer of xylitol but also the rate limiter.[7−10]

In this work, we aimed at solving the problems persecuting the microbial xylitol production methods. Since the overall process is limited by the reduction of D-xylulose to xylitol, we purified and characterized the enzyme responsible for this step from *G. oxydans* NH-10, the xylitol dehydrogenase. Sequences of both the protein and the nucleotide fragment leading to the catalytic activity exhibited a high correlation with xylitol dehydrogenase from *G. oxydans* 621H. Moreover, the XDH showed some common enzymatic properties to the XDHs from *G. oxydans* ATCC 621, *Candida shehatae*, and *Pachysolen tannophilus*,[26,27] such as the optimal pH, temperature, and relevant stability and coenzyme specificity.

As another key individual enzyme of the bioconversion process, the membrane-bound D-arabitol dehydrogenase (m-ArDH) is responsible for catalyzing a complete conversion.[8] Comparison of the enzyme activity, cofactor availability, and localization[27,28] proved to be a concise way toward understanding the conversion efficiency differences between m-ArDH and XDH. Previous studies revealed that the specific activity of m-ArDH was about 10-fold greater than that of XDH, with their optimal substrates. Furthermore, the reduction by XDH only took place when the cofactor was sufficient and cycling, while that by m-ArDH proceeded in a different way where electrons would be transferred via ubiquinone which is directly related to the respiratory chain. In conclusion, XDH catalyzes the rate-limiting step and thus determines the rate of overall transformation process.

So far, no attempts have been taken to work along both lines in *G. oxydans*, the XDH enzyme activity and its cofactor supply. This study is the first to utilize coexpression system in the wild *G. oxydans* strain in order to improve xylitol production from D-arabitol. Working as a broad-host-range plasmid with a multicopy site, the vector system pBBR1MCS-5 was often used to harbor genes coding for the dehydrogenases that are involved in the corresponding production processes.[29−31] The *G. oxydans tufB* promoter, i.e., the promoter of the elongation factor TU, was always able to elicit the greatest increase of enzyme activity and some other favorable physiological properties.[30,31] Therefore we chose the system pBBR1MCS-5 and promoter P_{tufB} to realize the genetic manipulation of the wild *G. oxydans*.

Physiological status of the resulting recombinant strains confirmed that the expression vector with individual genes does not affect the host significantly. The conversion rate of D-arabitol to xylitol by the coexpression strain was also increased by a wide margin. When D-glucose was added into the reaction system as the cosubstrate, GDH would ameliorate the recycle of NAD^+/NADH so as to maintain a suitable quantity of cofactors for the sugar alcohol conversion. Meanwhile, the overproduced XDH catalyzed D-xylulose transformation at a higher rate and larger quantity of xylitol accumulated due to the adequate concentration of NADH. From the discussion, one may conclude that the recombinant strain with coexpression plasmids enhanced the XDH/GDH activities as well as the NADH regeneration without affecting the regular growth levels, displaying more potential in the conversion of xylitol from D-arabitol.

Afterward, transverse comparison and analysis of the results of the similar researches were conducted. The xylitol yield in this study is higher than that of the engineered *G. oxydans* reported by M. Sugiyama,[27] which was already 11-fold more active than the wild type, implying that close attention paid to the XDH activity alone is not enough. S. Suzuki[7] investigated the effect and mechanism of exogenous addition of different cosubstrates on the production of xylitol and concluded that cosubstrate worked by increasing the supply of NADH, laying down the base for our further manipulating the cofactor regeneration. On top of that, this recombinant strain here possesses some superiority over the engineered *E. coli* with coexpression systems constructed by P. Zhou[10] in the sense of

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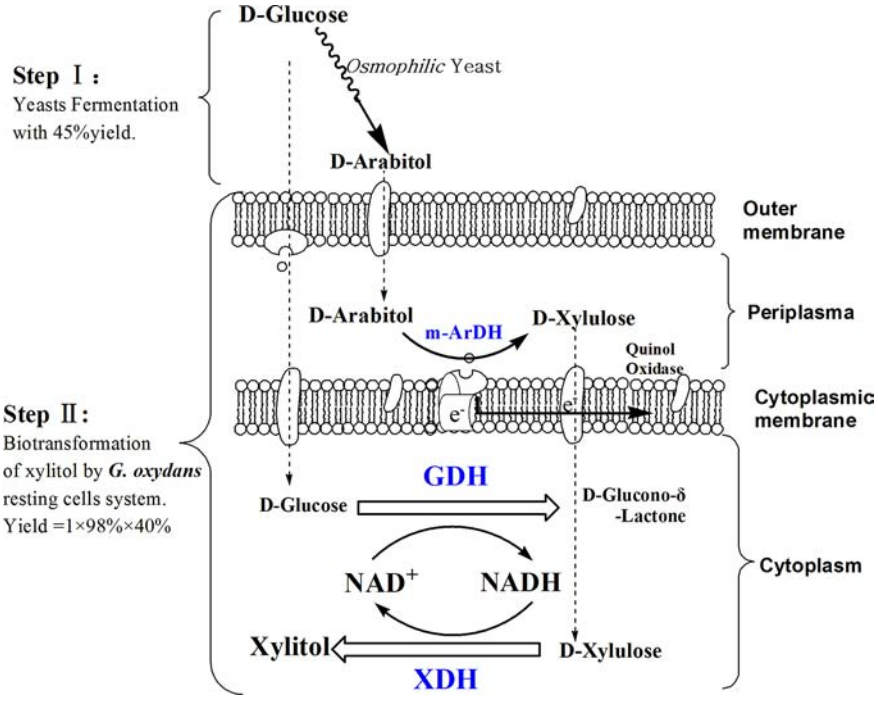

Figure 5. Schematic diagram of bioproduction pathway of xylitol originated from D-glucose with the *G. oxydans* PXPG.

safety and operational complexity. It is believed that our genetic modification of *G. oxydans* is a powerful tool for improving xylitol productivity.

A schematic illustration of the production pathway from D-glucose to xylitol within the bioconversion is shown in Figure 5. Although the productivity of xylitol has been enhanced by the introduction of recombinant plasmids, the current method is far from perfect for large-scale production of xylitol. Further progress could be made by (i) optimization of the expression systems or attempts of integrant expression to obtain remarkable activity promotion; (ii) modification of the metabolic pathways involved in coenzyme regeneration. In addition, it will be promising to apply the cell immobilization method for the purpose of continuous bioconversion and reutilization of the biocatalytic agent.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Abs*, absorbance at * nm; CGMCC, China general microorganism culture center; SRM, sugar-rich medium; HPLC, high-performance liquid chromatography; IPTG, isopropyl *b*-D-1-thiogalactopyranoside; KDa, kilodalton; KPB, potassium phosphate buffer; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PQQ, pyrroloquinoline quinine; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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