0273-2289/98/70–72—0405$10.50

The Production and Properties of a New Xylose Reductase from Fungus *Neurospora crassa*

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ABSTRACT

Neurospora crassa X1 was found to ferment xylose and glucose simultaneously. Xylose was the appropriate inducer for the production of xylose reductase that had two isoenzymes designated as EI and EII. Both EI and EII, which were purified by affinity chromatography, had NADPH-dependent xylose reductase activities. EII also had NADH-dependent activity, and EI is the only xylose reductase found so far without any NADH-dependent activity. EI and EII had MWs of 30 kDa and 27 kDa, and p*I*s of 5.6 and 5.2, respectively. The specifities of EI and EII against triose, pentoses, and hexoses were studied. The K_ms against xylose for EI and EII were 2.3 m*M* and 1.1 m*M* respectively, which were much lower than those of the xylose reductase from yeast.

Index Entries: Xylose; xylose reductase; purification; characterization; *Neurospora crassa*.

INTRODUCTION

Xylose is the main sugar component of hemicellulose which comprises 30–40% in some agriculture residues such as corn stover and sugarcane *(1)*. Bacteria *(2)* and yeasts *(3)* have quite different ways to use xylose. Since xylose-isomerase gene from bacteria did not express sufficiently in yeasts, the studies of xylose utilization have been focused on the xylose metabolic pathway in yeasts such as *Candida, Pichia,* and *Pachysolen.* The characteristics of xylose reductase (EC 1.1.1.21) in yeasts vary between species and strains. *Pichia stipitis (5)* and *Candida (6)* species have only

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a single xylose reductase that has both NADPH- and NADH-dependent activities. The xylose reductase from *Pachysolen tannophilus* has two isoenzymes. Verduyn *(7)* indicated that one of these isoenzymes had both NADPH- and NADH-dependent xylose reductase activities, and the other one had NADPH-dependent activity with very low NADH-dependent activity. However, Ditzelmuler *(8)* reported that both of the isoenzymes from *Pachysolen tannophilus* had NADPH- and NADH-dependent activities. But all these yeasts can not utilize xylose in cellulosic hydrolysate efficiently.

Efforts have been made to look for microorganisms that can directly convert cellulosic materials to ethanol. *Neurospora crassa*, a well-studied fungus in genetic and molecular biology, is found to have the ability to not only produce cellulase and hemicellulase, but also to use xylose and glucose when it grows on cellulosic materials. The studies of the cellulase and hemicellulase from *N. crassa* has been reported *(9,10)*, but its ability to use xylose and the enzymes involved in its xylose metabolism have not been investigated. Our studies focused on the xylose fermentation as well as the production and characterization of xylose reductase, the first enzyme, in the xylose metabolic pathway in *N. crassa* X1.

MATERIALS AND METHODS

Microorganism

Neurospora crassa X1 was isolated from decayed corn stover.

Medium

Nutrient salts for liquid medium: KH_2PO_4 2.0 g, $(NH_4)_2SO_4$ 1.4 g, urea 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $CaCl_2$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, MnSO H_2O 1.5 mg, $ZnSO_4$ 1.4 mg, $CoCl_2$ 2.0 mg, dissolved in 1000 mL dH_2O. pH was adjusted to 5.5 with HCI or NaOH.

Liquid medium for glucose and xylose utilization contained 2.4% glucose, 1.6% xylose, and nutrient salts. Medium for the production of xylose reductase contained 1% xylose and nutrient salts.

To study the influence of sugars on the production of xylose reductase, 2% glucose, xylose, galactose, mannose or arabinose was used respectively as carbon source, and 0.1% one of the other four sugars was supplemented for the study of the induction of this sugar to xylose reductase. Sugars were dissolved in the nutrient salts solution described above.

Culture Condition

About 10^6 fresh spores of *N. crassa* X1 from 5 days' potato-agar slant were inoculated in 50 mL liquid medium, cultivated at 30°C with shaking under 100 rpm.

Preparation of Cell-Free Extract

Mycelia of *N. crassa* X1 were collected from 50 ml, 3 days' liquid culture, washed twice with water, resuspended in 5 mL of pH 7.2, 0.05 *M* phosphate buffer containing 1 m*M* 2-mercaptoethanol, then ground with quartz sand in a T-shaped motor grinder tube for 5 min at 10*g*. Whole cells and debris were removed by centrifugation at 10,000*g*, for 30 min, at 4°C. The supernatant was collected as crude xylose reductase.

Determination

Enzyme Assay (6)

Xylose reductase activity was assayed by mixing 0.1 mL diluted enzyme solution with 0.1 mL of 1 *M* xylose and 0.7 mL of 0.1 *M*, pH 7.2 phosphate buffer, then adding 0.1 mL of 3.4-m*M* NADPH (or NADH). This solution was immediately mixed and the absorbance (OD) at 340 nm vs time of reaction was recorded at 20°C. One unit (U) of the enzyme activity was defined as one μmol NADPH (or NADH) oxidized in one minute. Specific activity was expressed as U/mg protein.

For the studies of substrate specificities of EI and EII, xylose in the assay was replaced by D/L-glyceride, arabinose, ribose, mannose, galactose, glucose, or 2-D-deoxy-glucose, respectively.

Glycoprotein (12)

Diluted enzyme solution (1 mL) was mixed with 1 mL 2.5% phenol, 5 mL 98% H_2SO_4, and kept at room temperature for 30 min. Then the absorbance of this solution at 490 nm was determined. Glucose was used as a standard, and the concentration of sugar in protein was calculated as glucose.

Protein (13)

Soluble protein was determined by Folin-phenol method with bovine serum albumin as a standard.

Amino Acid (14)

Tyrosine and tryptophan contents *(M)* were determined by keeping a mixture containing 1.0 mL enzyme solution and 1.0 mL, 0.2 *N* NaOH at 30°C for 3 h. Then the absorbances *(A)* at both 294.4 nm and 280 nm were recorded for the calculations of tyrosine and tryptophan as follows:

$$M_{\text{Tyrosine}} = (0.592 \times A_{294.4} - 0.263 \times A_{280}) \times 10^{-3}$$

$$M_{\text{Tryptophan}} = (0.263 \times A_{280} - 0.170 \times A_{294.4}) \times 10^{-3}$$

Other amino acids were determined on the amino acid analyzer. The

protein was hydrolyzed by mixing 1 mL protein solution with 1 mL, 6 *N* HCL, and hydrolyzing at 110°C for 20 h in N_2. Then the hydrolysate was evaporated, redissolved in 0.02 *N* HCL, and put on the amino acid analyzer (Beckman, HPLC equipped with Waters, amino acid analysis column).

Enzyme Purification

Affinity Chromatography

A column packed with reactive red agarose-120 gel (Type 3000-CL, Sigma) was equilibrated with pH 7.2, 0.05 *M* potassium phosphate buffer before crude xylose reductase was loaded. The loaded column was eluted with equilibration buffer, 0.5 *M* and 2.0 *M* KCl salt solutions, subsequently. The two salt-eluted fractions were collected, dialyzed against 0.05-*M*, pH 7.2 potassium phosphate buffer and assayed for xylose reductase activity. The purification was carried out at room temperature.

HPLC

Purified xylose reductase fractions from affinity chromatography were applied to a pre-equilibrated ion-exchange column on Waters-991 HPLC. Elution was carried out with pH 7.2, 0.05 *M* potassium phosphate buffer and then with a 0.1–1.0 *M* NaCl gradient at a rate of 1.0 mL/min. Peaks were detected at 280 nm.

Glucose and xylose were also determined by HPLC.

SDS-Gradient Ultra-Thin PAGE

Polyacrylamide gradient gel (3.9–22.5%) was used for SDS-electrophoresis on Pharmacia (Uppsala, Sweden) LKB MultiDive XL Type-Multiphor II Type equipment, under 100 V, 15′ and 600 V, 20A. Standard proteins were bovin (67,000), egg albumin (45,000), glycerol aldehyde 3-p-dehydragenase (36,000), carbonic anhydrase (30,000), trypsinogen (23,000), trypsin inhibitor (22,500). and α-lactalbumin (14,000) (Sigma).

IEF

Gradient gel (pH 3.5–9.5) was used in isoelectrofocusing at 50 mA, 20 W. The same equipment was used as in SDS-PAGE. Standard proteins were soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.2), bovin carbonic anhydrase B (5.85), human carbonicanhydrase B (6.55), horse myoglobin (6.85, 7.35), lenti (8.15, 8.65), and trypsinogen (9.3) (Sigma).

RESULTS AND DISCUSSIONS

Utilization of Xylose in the Presence of Glucose

Assimilation of glucose and xylose by *N. crassa* X1 was studied in liquid medium containing glucose and xylose. Results shown in Fig. 1

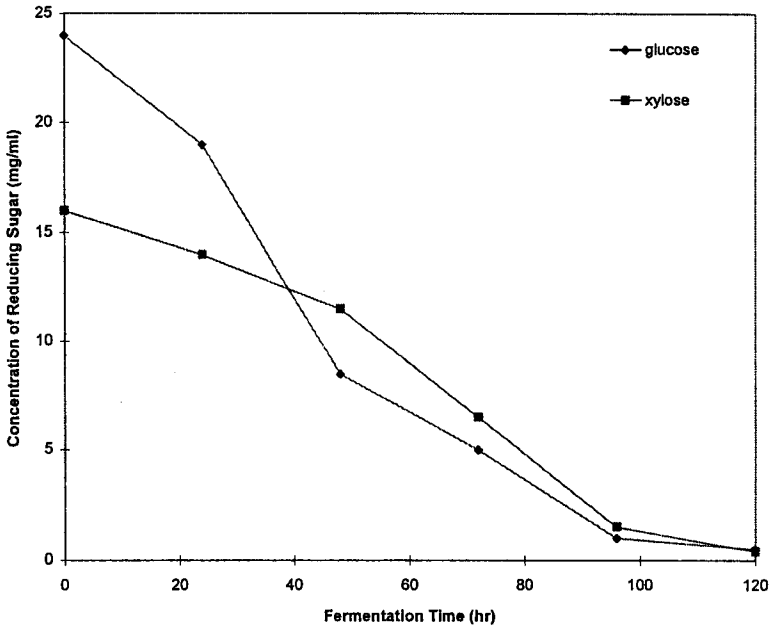

Fig. 1. Utilization of xylose in the presence of glucose by *N. crassa* X1.

indicated that *N. crassa* X1 had quite different characteristics from those of yeasts. *N. crassa* X1 can use glucose and xylose simultaneously according to Fig. 1, although the utilization of xylose was slower than that of glucose. However, in yeasts *(6)* such as *Candida Pichia*, and *Pachysolen*, when glucose and xylose presented in the media at the same time, xylose will not be consumed until glucose was used up, and a retention time of approx 10 h was needed before the assimilation of xylose. Similar to yeasts, *N. crassa* X1 converted glucose and xylose to ethanol and xylitol.

Production of Xylose Reductase

N. crassa X1 was cultivated in 50 mL, 1% xylose liquid medium. Samples were taken to have the xylose reductase activities and remained xylose examined. Results in Fig. 2 showed that the xylose in medium was almost used up after 96 h, and xylose reductase reached to its highest levels of approx 220 U/mL for NADPH-dependent activity, and 100 U/mL for NADH-dependent activity.

Influence of Carbon Sources on the Production of Xylose Reductase

Results of the influence of carbon sources on the production of xylose reductase from *N. crassa* X1 are presented in Table 1. Comparing the enzyme activities from cells growing in xylose with those growing in glucose or arabinose, the highest levels of xylose reductase specific activities were obtained only from the cells growing in xylose, which were 111 U/mg

Table 1
Effects of Different Carbon Sources on the Production of Xylose Reductase from *N. crassa* X1

Carbon Source	Specific Xylose Reductase Activity			
	NADPH dependent		NADH dependent	
	U/ml	U/mg.protein	U/ml	U/mg.protein
2% glucose	72	34	24	11
+0.1% galactose	80	33	44	18
+0.1% arabinose	128	58	40	18
+0.1% mannose	36	37	80	33
+0.1% xylose	88	44	64	32
2% xylose	344	111	68	21
+0.1% galactose	310	111	61	22
+0.1% arabinose	352	117	48	16
+0.1% mannose	368	119	80	25
+0.1% glucose	302	104	60	21
2% arabinose	136	62	44	20

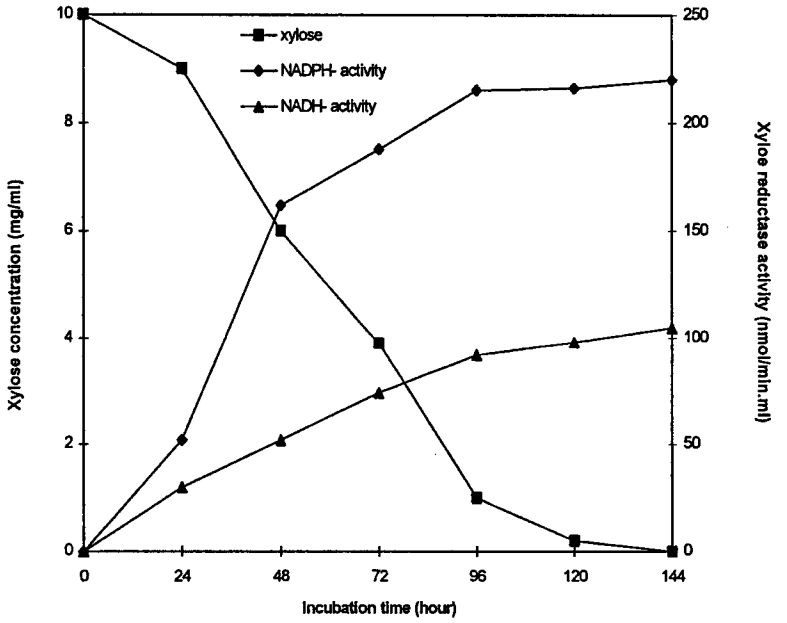

Fig. 2. The production of xylose reductase from *N. crassa* X1 in 1% xylose liquid culture.

protein for NADPH-dependent and 21 U/mg protein for NADH-dependent activities. The NADPH-dependent activity from cells growing in arabinose was about half of that in xylose. However, in *Pachysolen (15)* it was reported that the xylose reductase activity from cells growing in arabinose was two times higher than that from cells growing in xylose.

From *N. crassa* X1 growing in 2% glucose, 34 U/mg protein of NADPH-dependent as well as 11 U/mg protein of NADH-dependent reductase activities against xylose were obtained. However, it is reported for *Pachysolen (15)* that no xylose reductase activities were detected from cells growing in glucose.

From the results above, the production of xylose reductase from *N. crassa* X1 seemed not to be strictly inducible by pentose, and this may result in its simultaneous utilization of glucose and xylose (Fig. 1). This characteristic is quite different from yeasts, and might be useful in the fermentation of hydrolysate from cellulosic materials that contain glucose and xylose.

Purification of Xylose Reductase from *N. crassa* X1

Two fractions of xylose reductase, designated as EI and EII, were obtained from *N. crassa* X1 cell extract after the purification with affinity chromatography (reactive red agarose). EI, which was the fraction eluted with 0.5 *M* KCL, only had NADPH-dependent xylose reductase activity. EII, which was eluted with 2.0 *M* KCL, had both NADPH-dependent and NADH-dependent activities. HPLC was also used for further purification of EI and EII. The molecular weights of EI and EII, which were obtained from SDS-PAGE, were 30 kDa and 27 kDa, respectively. The pIs of EI and EII, which were obtained from electrofocusing, were 5.3 and 5.6, respectively.

Some of these characteristics of xylose reductase from *N. crassa* X1 were different from those reported from yeasts. First, all yeasts except *Pachysolen* had a single xylose reductase component; *Pachysolen* had two isoenzymes *(7,8)*, both of which had NADPH-dependent and NADH-dependent activities. However, we found two isoenzymes (EI and EII) in *N. crassa* X1. EII had both NADPH- and NADH-dependent activities, but EI had no NADH-dependent activity. Since EI was the only xylose reductase found so far without NADH-dependent activity, it may be used in studying the mechanism of NADH regeneration in pentose pathway to improve the enthanol production. Second, as reported, the molecular weights of xylose reductase from yeasts ranged from 31 kDa *(18)* to 70 kDa *(6)*, whereas EI and EII had MWs below 30 kDa. Finally, the pIs for yeasts were reported as 4.6 *(8)* and 5.2 *(18)*, which were lower than those (5.3 and 5.6) of EI and EII.

Determination of Glycoproteins and Amino Acid Composition of EI and EII

Both EI and EII were glycoproteins. The sugar contents (calculated as glucose) of EI and EII was 7.57% (w/w) and 11.3% (w/w) respectively.

The amino acid composition of EI and EII is shown in Table 2. The results indicate that the acidic amino acid contents of EI and EII were 20.5

Table 2
Amino Acid Composition of the Xylose Reductase EI and EII from *N. crassa* X1

Amino acid[a]	EI (mol%)	EII (mol%)	Amino acid	EI (mol%)	EII (mol%)
Asx[b]	9.55	9.31	Leu	10.00	10.99
Thr	5.66	4.75	Tyr[d]	3.00	3.37
Ser	5.69	6.06	Phe	3.38	4.49
Glx[c]	10.94	11.27	Trp[d]	1.22	1.36
Gly	9.00	8.17	Lys	7.23	6.81
Ala	8.93	8.37	His	2.20	2.14
Val	7.34	6.37	Arg	4.49	5.24
Met	0.45	0.125	Pro	4.89	5.10
Ile	5.57	5.81			

[a] Trace Cys was detected.
[b] Asx = Asn + Asp.
[c] Glx = Gln + Glu.
[d] Tyr and Trp were analyzed by spectrophotometer.

and 20.6%, respectively, which are much lower than that of xylose reductase from *Candida shehatae (6)*, which is 35%.

Substrate Specificity of EI and EII

Both EI and EII exhibited Michaelis-Menten kinetics with respect to their substrates. K_m value was calculated from the double reciprocal plots of v^{-1} vs [xylose]$^{-1}$, v^{-1} vs [NADPH]$^{-1}$, and v^{-1} vs [NADH]$^{-1}$ which were based on Michaelis-Menten equation *(19)*. [xylose] *(M)*, [NADPH] (m*M*) and [NADH] (m*M*) represent the concentrations of xylose, NADPH and NADH, respectively. Velocity *(v)* is expressed as the decrease of [NADPH] or [NADH] (m*M*) per minute. The concentration of substrate tested was up to 0.5 *M* xylose and 0.5 m*M* NADPH or NADH.

Eight sugars, listed in Table 3, were employed to determine the substrate affinity (K_m) of NADPH-dependent xylose reductase from *N. crassa* X1. The results were also compared with those of yeasts. In Table 3, the lowest K_m of 1.1 m*M* against xylose was obtained from EII. However, EI and the xylose reductases from yeasts had the lowest K_ms against D/L-gluceride instead of xylose. This indicated that only EII had the highest binding affinity on xylose. Therefore, EII might be considered a strict xylose reductase, and the other xylose reductases in Table 3 might be only regarded as aldose reductases.

Also from Table 3, when xylose was used as the substrate, EI and EII had K_ms of 2.3 m*M* and 1.1 m*M* against xylose, respectively, which were much lower than those of yeasts. This implies that the xylose reductases from *N. crassa* X1 (EI, EII) have a much higher binding affinity on xylose than those from yeasts.

Table 3
Comparison of Substrate Specificity of NADPH-Dependent Xylose Reductase from *N. crassa* X1, *P. stipitis*, *P. quercunm*, *C. utilis*, and *P. tannophilus*

	Km (mM) (NADPH-dependent activity)					
	N. crassa EI	*N. crassa* EII	*Pi. stipitis*	*Pi. quercunm*	*C. utilis*	*Pa. tannophilus*
Substrate						
D.L-glyceride	0.7	36	18			1.7
xylose	2.3	1.1	42	78	28	12
arabinose	53	184	40			18
ribose	95	122	310		67	
mannose	>1000	NU				200
galactose	>1000	>1000	140			25
glucose	>1000	>1000	420			
2-D-deoxy-glucose	>1000	NU				33
Reference	This work	This work	7	5	5	16

Note: NU=no utilization

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CONCLUSION

In conclusion, *N. crassa* X1 assimilated glucose and xylose simultaneously, and xylose reductase can be detected from cells growing in both pentose and hexose. Its combined characteristics of the utilization of cellulose/hemicellulose and glucose/xylose in a single strain will be very useful in investigating the mechanism of direct conversion of cellulosic materials to ethanol.

The xylose reductase from *N. crassa* X1 has two isoenzymes (EI and EII). EI is the only xylose reductase found so far without any NADH-dependent activity. EII has the highest binding affinity on xylose, and may be considered a strict xylose reductase. Further kinetics studies on EI and EII will be carried out to reveal the binding sites of xylose, NADPH, and NADH on the enzyme.

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