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Activity of Xylose Reductase from *Candida mogii* Grown in Media Containing Different Concentrations of Rice Straw Hydrolysate

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Abstract

Xylose reductase (XR) activity was evaluated in extracts of *Candida mogii* grown in media containing different concentrations of rice straw hydrolysate. Results of XR activity were compared to xylitol production and a similar behavior was observed for these parameters. Highest values of specific production and productivity were found for xylose reductase (35 U/g of cell and 0.97 U/[g of cell·h], respectively) and for xylitol (5.63 g/g of cell and 0.13 g/[g of cell·h]) in fermentation conducted in medium containing 49.2 g of xylose/L. The maximum value of XR:XD ratio (1.82) was also calculated under this initial xylose concentration with 60 h of fermentation.

Index Entries: *Candida mogii*; hydrolysate concentration; rice straw; xylitol; xylose reductase.

Introduction

Xylose reductase (XR) is the enzyme responsible for the first step in xylose metabolism by yeasts *(1)*. In a reaction catalyzed by this enzyme, xylose is reduced to xylitol, which can be oxidized into xylulose or released into the environment, depending on the culture conditions of the microorganism. The oxidation of this polyalcohol is catalyzed by the enzyme xylitol dehydrogenase (XD). Xylitol is a sugar alcohol of economic interest because of its dietetic and anticariogenic properties *(2,3)*. Microbial and enzymatic processes have been studied as alternatives to the chemical

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process currently employed for its production *(4–8)*. These biologic processes are expected to reduce the final cost of xylitol by allowing the utilization of xylose from hydrolysates of lignocellulosic materials, thus eliminating the need for the previous purification of this sugar *(9)*. Among several residues utilized as a xylose source for xylitol production by yeasts, rice straw has been deemed a potential substrate for this bioconversion *(6,9)*. Many yeasts have the capability of efficiently producing xylitol from hemicellulose hydrolysates. In a previous study, *Candida mogii* NRRL Y-17032 was selected among 31 yeast strains as a promising xylitol producer from rice straw hemicellulose hydrolysate *(10)*. Compared to microbiologic fermentation processes, the approach by enzyme technology employing isolated XR for xylitol synthesis should make a substantial increase in productivity because mass transfer limitations are avoided in homogeneous enzyme reactors *(7)*. Taking into account that XR is an inducible enzyme, enhancing its production depends on the optimization of microorganism culture conditions. Some factors have been studied in order to evaluate their influence on XR activity *(11,12)*. In the present study, the effect of initial substrate concentration was investigated in *C. mogii* fermentations employing rice straw hydrolysate as the source of carbon.

Materials and Methods

Preparation of Hemicellulose Hydrolysate

Rice straw hemicellulose hydrolysate was obtained by acid hydrolysis of the rice straw in an AISI 316 stainless steel 350-L stirred-tank reactor. The hydrolysis was run for 20 min at 120°C using 10 mL of 0.13 *M* H_2SO_4 solution/g of dry matter. The obtained hemicellulose hydrolysate was collected by centrifugation and homogenized. The following components were detected: 18.1 g/L of xylose, 3.5 g/L of glucose, 2.8 g/L of arabinose, 0.025 g/L of furfural, and 0.038 g/L of hydroxymethylfurfural (HMF). This hydrolysate was concentrated under vacuum at 70°C and the xylose concentration reached 220 g/L. Next, the pH was raised with NaOH pellets up to 8.0 and then lowered to 6.0 with 72% H_2SO_4 (w/w). Each time the pH level was changed, the precipitate was removed by centrifugation (2860*g* for 30 min).

Microorganism and Preparation of Inoculum

C. mogii NRRL Y-17032 obtained from Northern Regional Research Laboratory (Peoria, IL) was maintained at 4°C on nutrient agar slants. Inoculum was prepared by cultivating cells in 125-mL Erlenmeyer flasks containing 25 mL of medium. The medium was composed of rice straw hemicellulose hydrolysate diluted with distilled water to provide an initial xylose concentration of 30 g/L. Loopfulls of cells were suspended in a few milliliters of distilled water, and this suspension was pipetted into the flasks. The flasks were incubated in a rotary shaker at 200 rpm and 30°C. After 48 h, cells were harvested by centrifugation. The fermentation medium contained an initial cell concentration of 1 g/L.

Fermentation Conditions

Fermentation media were composed of rice straw hemicellulose hydrolysate containing 220 g/L of xylose diluted with distilled water to provide initial xylose concentrations of 30, 40, 50, 70, and 90 g/L. Twenty-five milliliters of these media was placed into 125-mL Erlenmeyer flasks that were inoculated and then incubated at 30°C at 200 rpm. Each sample was constituted by the total volume of one Erlenmeyer flask.

Preparation of Cell-Free Extracts

Cells were harvested by centrifuging at 2860*g* for 30 min at 4°C, washed with 0.1 *M* potassium phosphate buffer (pH 7.2), and resuspended to a concentration of 15 g of dry cell weight/L with the same buffer. Cell disruption was conducted by sonication for 35 min in 1-s pulses with 1-s intervals in Sonics & Materials Disrupter equipment. Samples were centrifuged for 10 min at 6000*g* in a Jouan MR1812 centrifuge, and the cell-free extract was used for enzymatic tests.

Xylose Reductase Assay

Enzyme activities were determined spectrophotometrically by following the oxidation or reduction of the coenzyme at 340 nm. The assays were performed as described by Bolen and Detroy *(13)* except for cofactor concentration and buffer pH. The XR assay reaction mixture contained 50 m*M* potassium phosphate buffer, pH 7.2; 10 m*M* mercaptoethanol; 0.12 m*M* NADPH; 50 m*M* D-xylose; cell extract; and distilled water in a total volume of 1 mL. The reaction was started by the addition of D-xylose. XD activity was determined in a similar manner with phosphate buffer, D-xylose, and NADPH substituted by 75 m*M* Tris buffer, pH 8.6; 50 m*M* xylitol; and 0.12 m*M* NAD^+, respectively. One unit was defined as the amount of enzyme catalyzing the oxidation or reduction of 1 μmol of cofactor/min. Specific activities were expressed as units per gram of cell.

Analytical Methods

Cell mass was determined by using a calibration curve that correlates optical density at 600 nm and dry cell weight. Concentrations of sugars were determined by high-performance liquid chromatography (HPLC) using a Shimadzu C-R7A chromatograph equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column. Concentrations of furfural and HMF were determined by HPLC using a Waters chromatograph equipped with an ultraviolet detector and an RP18 column.

Results and Discussion

Table 1 presents the partial composition of fermentation media. Xylose concentrations were close to those expected from the dilutions, varying from 29.2 to 88.3 g/L. Concentrations of glucose and arabinose were both

Table 1
Partial Composition of Fermentation Media
with Hydrolysate Concentrations Equivalent to Different Xylose Contents

Component (g/L)	Xylose content (g/L)				
	29.2	38.6	49.3	69.8	88.3
Glucose	5.5	7.3	9.2	13.0	15.6
Arabinose	5.1	6.7	8.6	12.1	14.6
Furfural	0.017	0.021	ND[a]	ND[a]	0.028
HMF	0.026	0.032	0.042	0.062	0.076

[a]ND, not determined.

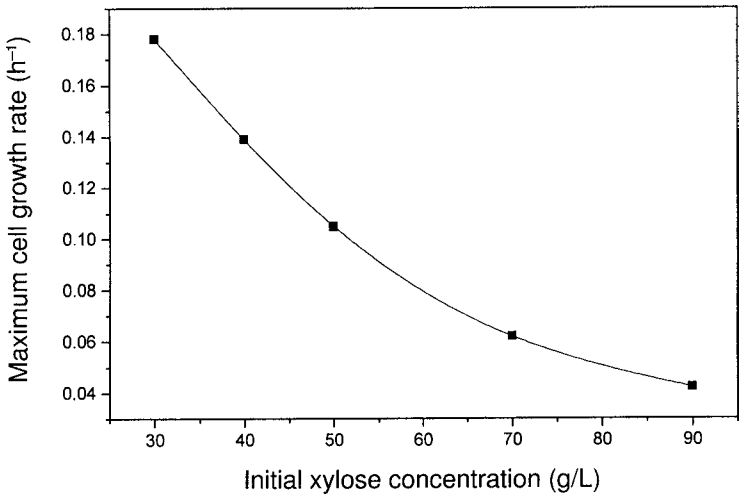

Fig. 1. Effect of hydrolysate concentration expressed as initial xylose content on maximum cell growth rates of the yeast *C. mogii*.

about 18% in relation to xylose levels. In addition to sugars, other substances could also be detected. Furfural and HMF are frequently found in hemicellulose hydrolysates *(14,15)*. Acetic acid and lignin degradation products were not analyzed and may have volatilized during vacuum concentration. Initially, we intended to study a range of hydrolysate concentrations larger than that presented. However, in another study for xylitol production employing the yeast *Candida guilliermondii* and rice straw hydrolysate *(16)*, no significant substrate consumption was detected above 90 g/L of xylose. Because the yeast *C. mogii* has presented a behavior similar to *C. guilliermondii* in all the studies conducted previously *(10)*, the range was restricted up to 90 g/L.

Figure 1 shows maximum cell growth rates. This parameter was strongly influenced by rice straw hydrolysate concentration. A gradual decrease in maximum cell growth rate resulted by increasing hydrolysate

Table 2
Fermentative and Enzymatic Parameters Determined Along Fermentation Time with Initial Rice Straw Hydrolysate Contents Equivalent to Different Xylose Concentrations

S_0 (g/L)	Run time (h)	Xylose used (%)	$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	q_{P1} (g/[g·h])	q_{P2} (U/[g·h])	P_{xtol} (g/g)	P_{XR} (U/g)	XR:XDH ratio
29.2	24	84	0.43	0.19	0.09	0.79	2.23	19	0.69
	36	97	0.42	0.24	0.05	0.36	1.78	13	0.89
	48	97	0.39	0.32	0.03	0.15	1.24	7	1.02
38.6	24	69	0.47	0.17	0.11	0.46	2.74	11	0.88
	36	93	0.46	0.16	0.08	0.56	2.95	20	0.81
	48	97	0.44	0.22	0.04	0.33	2.03	16	0.80
49.3	36	82	0.59	0.13	0.13	0.97	4.64	35	1.70
	48	95	0.58	0.10	0.12	0.63	5.55	30	1.29
	60	99	0.54	0.10	0.09	0.50	5.63	30	1.82
69.8	48	37	0.65	0.15	0.09	0.21	4.32	10	1.38
	72	66	0.53	0.11	0.07	0.18	4.82	13	0.58
	96	99	0.45	0.09	0.05	0.22	5.01	21	0.75
88.3	96	43	0.32	0.09	0.04	0.16	3.79	15	1.75
	120	49	0.30	0.12	0.02	0.08	2.60	9	1.68
	144	65	0.24	0.08	0.02	0.08	3.25	11	1.51

[a]$Y_{P/S}$, xylitol yield (g/g of xylose used); $Y_{X/S}$, cell yield (g/g of xylose used); q_{P1}, specific xylitol productivity (g/[g of cell·h]); q_{P2}, specific XR productivity (U/[g of cell·h]); P_{xtol}, specific xylitol production (g/g of cell); P_{XR}, specific XR production (g/g of cell). Values are average of duplicates.

concentrations. The highest value (0.18 h^{-1}) was achieved with an initial xylose concentration of 29.2 g/L, and the lowest value (0.04 h^{-1}) was found in fermentation with 88.3 g of xylose/L. Several researchers have reported growth inhibition by rising substrate concentration on yeast physiology. A decline of yield and specific rate of cell production when the amount of xylose initially present in the culture increased was found in a study for xylitol production from *C. guilliermondii* in synthetic medium *(17)*. The yeasts *C. guilliermondii* and *Candida parapsilosis* showed the same relationship between cell growth and xylose concentration under different aeration conditions *(11)*. Growth inhibition in fermentations using hemicellulose hydrolysates as substrate has been reported at lower levels of xylose concentration than in synthetic media *(16,18)*. du Preez et al. *(18)* observed an increase in maximum specific growth rate from 0.21 to 0.35 h^{-1} by diluting to half the hydrolysate concentration in fermentation medium. These results suggest that in the present study inhibition has occurred as a function of the increase in concentration of toxic compounds.

Table 2 shows the results of the fermentative and enzymatic parameters monitored for each initial xylose concentration along fermentation time. The highest values of specific production of xylitol (5.63 g/g of cell) and XR (35 U/g of cell) were found with an initial xylose concentration of

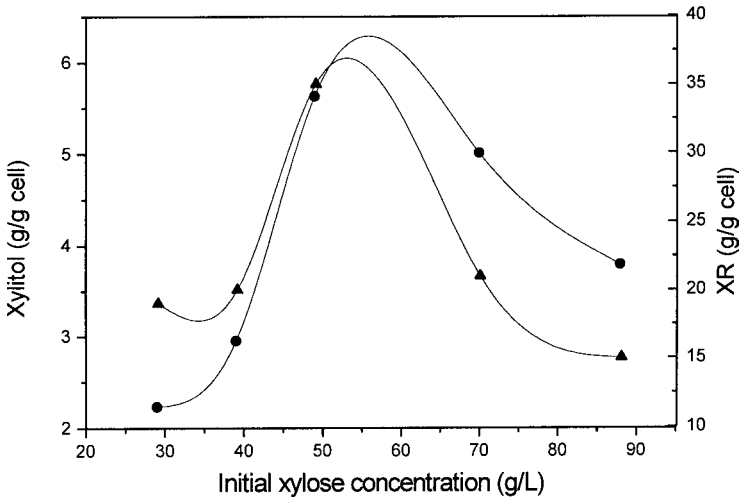

Fig. 2. Highest values of specific production for xylitol (—●—) and XR (—▲—) in fermentations with different hemicellulosic hydrolysate concentrations expressed as initial xylose content.

49.3 g/L. The lowest values for both xylitol (1.24 g/g of cell) and XR (7 U/g of cell) were determined at a fermentation condition of 29.2 g of xylose/L and 48 h. The effect of initial substrate concentration condition on specific production is shown in Fig. 2, where points of maximum can be observed at about 50 g of xylose/L. Time course profiles suggested a relationship between xylitol and XR for this parameter (Fig. 3). In most cases, values increased or decreased with xylitol-specific formation. Nolleau et al. *(11)* reported similar behavior when evaluating the influence of xylose concentration on xylitol production from strains of *C. guilliermondii* and *C. parapsilosis* in synthetic medium. According to them, maximum values of specific enzyme activity were found for *C. guilliermondii* (0.63 U/mg of total protein) and *C. parapsilosis* (0.42 U/mg of total protein) at their optimal initial xylose concentrations (100–150 and 200–300 g/L, respectively) for xylitol accumulation.

According to Table 2, values of specific productivity varied from 0.02 to 0.13 g/(g of cell·h) for xylitol and from 0.08 to 0.97 U/(g of cell·h) for XR. For this parameter, a relationship between xylitol and XR along the fermentation time was found only in fermentations with 29.2, 49.3, and 88.3 g of xylose/L. Figure 4 shows the effect of initial substrate concentration condition on specific productivity. The highest values for both products were found in fermentation with 49.3 g of xylose/L. Specific production rates of xylitol and XR have already been associated in *C. mogii* fermentation using synthetic media with an initial xylose concentration varying from 5.3 to 53 g/L, although values have not been presented *(12)*. In the present study, the maximum values for total formation of xylitol and enzyme were found with an initial xylose concentration of 49 g/L in a 60-h

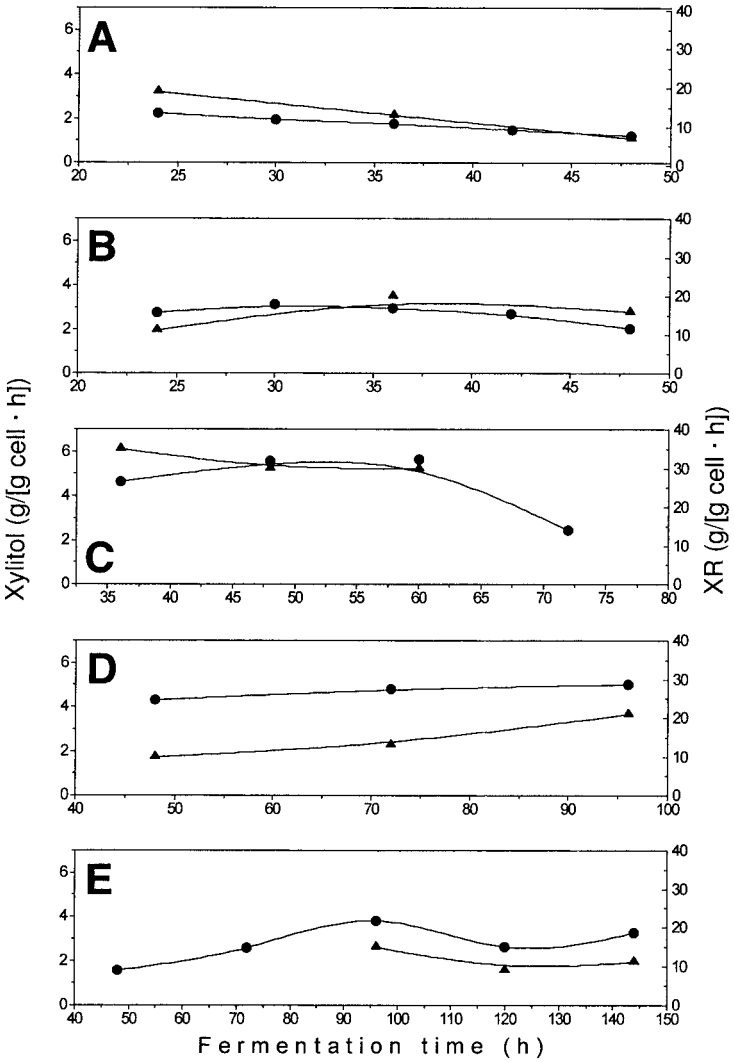

Fig. 3. Specific production of xylitol (—●—) and XR (—▲—) along fermentation time with hydrolysate concentrations equivalent to **(A)** 29.2, **(B)** 38.6, **(C)** 49.3, **(D)** 69.8, and **(E)** 88.3 g/L.

fermentation (data not shown). Under this condition, the XR:XD ratio was the highest (1.82). Similar values were calculated for the yeasts *Candida boidinii (19)* and *Debaryomyces hansenii (20)*. For these microorganisms, XR:XD ratio varied from 1.10 to 2.10 and 1.14 to 2.26, respectively.

Xylitol and XR production was positively influenced by initial hydrolysate concentration up to a certain level, whereas cell growth was strongly limited. Nevertheless, under higher initial hydrolysate concentrations, inhibition of xylitol and XR production was also observed. The inhibitory effect of hydrolysate concentrations in yeast fermentations has been attributed to toxic chemicals often formed during acid hydrolysis

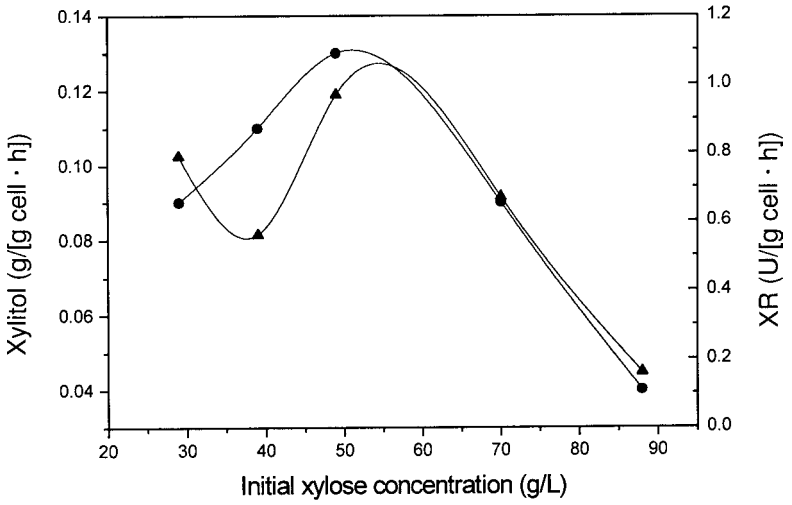

Fig. 4. Highest values of specific productivity for xylitol (—●—) and XR (—▲—) in fermentations with different hemicellulosic hydrolysate concentrations expressed as initial xylose content.

(16,21,22). In a study of the effects of lignocellulosic degradation products on xylose fermentation, Delgenes et al. *(23)* found that the intensity of growth inhibition was closely related to the initial concentration of tested inhibiting molecules.

Results drawn from the experiments in the present study showed an optimal initial hydrolysate concentration condition for xylitol and XR production with about 50 g of xylose/L. However, because the effect of substrate concentration seems to be dependent on aeration conditions *(11)*, it is possible that the formation of these products might be improved by using different aeration levels.

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