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Induction of aldose reductase activity in *Candida guilliermondii* by pentose sugars

Juliet Kiyoko Sugai[1]* and Jean-Philippe Delgenes[2]

[1]*University of Santa Catarina, Biological Science Center, Department of Biochemistry, Florianopolis-SC, Brazil and* [2]*National Institute of Agronomic Research, Laboratory of Environmental Biotechnology of Food Industry, Narbonne, France*

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SUMMARY

Cell extracts of *Candida guilliermondii* grown in D-xylose, L-arabinose, D-galactose, D-glucose, D-mannose and glycerol as sole carbon sources possessed NADPH-dependent aldose reductase activity, but no NADH-dependent activity was detected. D-xylose and L-arabinose were the best inducers of aldose reductase activity. The highest enzyme activity in D-xylose or L-arabinose-grown cells was observed first with L-arabinose followed by D-xylose as substrates of the enzymatic reaction. However, only low activity was found in D-glucose, D-mannose and D-galactose-grown cells, indicating that these carbon sources cause catabolite repression. Enzyme activities induced in D-xylose-grown cells were twice as high as those obtained from the cells under resting conditions. Furthermore, the level of induction of aldose reductase activity depended on the initial concentration of D-xylose. The present study shows that aldose reductase activity may be efficiently induced by pentose sugars of hemicellulosic hydrolysates and weakly by hemicellulosic hexoses.

INTRODUCTION

The abundant and renewable nature of agricultural residues and other biomass makes them of potential use as fermentation substrates. Hemicellulose is one of the major fermentable components of lignocelluloses, contributing between 15 and 35% of the dry weight biomass of some plant species [8]. Hydrolysis of hemicellulose yields a mixture of sugars with D-xylose as the major component, comprising nearly two-thirds of the reducing sugars, the remaining one-third being composed of L-arabinose, D-glucose, D-mannose and D-galactose [19]. The abundance and ease of isolation of xylose make it a potential source for the production of useful chemicals and liquid fuels such as xylitol and ethanol. A process for efficient xylose bioconversion is of economic interest because xylitol, a five-carbon polyalcohol, has several important uses: natural food sweetener, dental caries reducer, insulin-independent carbohydrate source for insulin-dependent diabetics, and rational treatment for other diseases such as erythrocytic glucose-6-phosphate dehydrogenase deficiency [10]. Xylitol is produced on an industrial scale through chemical reduction of D-xylose derived from hemicellulosic hydrolysates. The steps to purify xylitol from other polyols and by-product sugars makes its chemical production relatively expensive.

Xylitol can also be produced from D-xylose by many yeast species [2,14] involving NAD(P)H-dependent xylose reductase which reduces D-xylose to xylitol [3,5,7,22,26,27]. Xylose reductase, the first enzyme in the pathway, plays a regulatory role in D-xylose metabolism. The control of D-xylose utilization by yeasts is believed to be, in part, by the activity of aldose reductase, which is subject to regulation by induction and repression [3–5,17]. One aspect of this control is that xylitol-producing yeast strains, including *Pachysolen tannophilus* and *Candida boidinii*, utilize the pentoses slowly when more readily metabolized hexoses are present in the culture medium, as in the hydrolysates of plant biomass [3,9,28].

The present study was undertaken to investigate the effect of different carbon sources on the aldose reductase activity in *Candida guilliermondii*, which is characterized by a high potential for xylitol production from xylose-rich materials [2,20,21].

MATERIALS AND METHODS

Microorganism. Candida guilliermondii NRC 5578 was obtained from the Foundation for Industrial Technology (Sao Paulo, Brazil) and was maintained at 4 °C on slants of YM agar [29].

Preparation of inoculum and cultivation. An inoculum was prepared by transferring a loopful of cells from the YM agar medium slant into 100 ml of medium in a 250-ml Erlenmeyer flask. The culture medium was composed of 0.67% (w/v) yeast nitrogen base (YNB, Difco Lab., Detroit, MI, USA) without amino acids; 1.0% (w/v) yeast extract (Difco Lab.) and 2.0% (w/v) glycerol. The cells were incubated in a loosely capped

Correspondence to: J.-P. Delgenes, National Institute of Agronomic Research, Laboratory of Environmental Biotechnology of Food Industry, Narbonne, France.

*Present address: National Institute of Agronomic Research, Laboratory of Environmental Biotechnology of Food Industry, Narbonne, France.

250-ml Erlenmeyer flask at 30 °C on a shaker at 170 r.p.m. for 48 h (cell densities ranged between 2.2 and 2.5 g of dry cell weight per liter).

Culturing was performed in loosely capped 500-ml Erlenmeyer flasks containing 400 ml of the above mentioned medium, except that the glycerol was replaced by 2% of the individual hemicellulosic sugars. The carbon source solutions were autoclaved separately. Cultures were inoculated at 2% (v/v) and incubated at 30 °C on a rotary shaker at 170 r.p.m. The cells were harvested by centrifugation (2740 × *g* for 5 min at 4 °C) and washed twice with 150 mM KCl. The final cell pellets (3200 × *g*, 5 min) were stored at −20 °C until used for preparation of the cell-free extracts.

Enzyme induction. The induction of the aldose reductase by cells under resting conditions was carried out according to the following procedure: cells pregrown on 2% glycerol for 37 h were aseptically harvested by centrifugation (2740 × *g* for 5 min at 4 °C) and washed twice with sterile distilled water. Washed cells were resuspended in 100 mM phosphate buffer, pH 6.5 and starved for 1 h at 30 °C with agitation (170 r.p.m.). The cells were then harvested and resuspended in fresh phosphate buffer, resulting in a cell concentration of 4.0–5.5 g L^{-1} (dry wt). The induction of the enzyme was carried out by incubating the cells with a 4% D-xylose at a rotary shaking rate of 170 r.p.m. at 30 °C.

Preparation of cell-free extract. The cell pellet was resuspended to a concentration of 0.13 g wet cell weight ml^{-1} with 50 mM potassium phosphate buffer pH 7.0 containing 1 mM β-2-mercaptoethanol and the cells were disrupted with glass beads (0.425–0.600 mm, Sigma, St Louis, MO, USA) in a Retsch-MM2 disintegrator (Germany) at a vibration frequency of 1800 cycles min^{-1} for three 1-min bursts alternating with 2-min cooling intervals in an ice bath. The slurry was centrifuged at 28 000 × *g* for 15 min at 4 °C and the supernatant (cell-free extract) was used for enzyme assays.

Enzyme assays. The aldose reductase assay reaction mixture contained 50 mM potassium phosphate buffer (pH 6.0), 0.15 mM NAD(P)H, 100 mM D-xylose or L-arabinose and cell extract, in a total volume of 1 ml. The reaction was started by addition of substrate after equilibrating the reaction mixture to eliminate endogenous NAD(P)H consumption in crude extracts. The decrease in optical density was measured by the change in extinction at 340 nm at 23 °C. The enzyme activities were expressed in international units (U). One enzyme unit is defined as the amount of the enzyme which oxidized 1 μmol of NAD(P)H per minute at 23 °C under the conditions of the assay. In all assays, the reaction velocity was linearly proportional to the amount of cell-free extract added. Specific activities were expressed as U mg^{-1} of protein determined by the Coomassie Blue reagent based on the Bradford [6] method with bovine serum albumin as the standard.

Analytical methods. Growth was monitored by a dry weight method. Cell dry weight was determined by washing the cells remaining on a membrane filter (Millipore, Milford, USA, pore size, 0.22 μm) with distilled water and drying to constant weight at 105 °C. Sugars, polyols and glycerol concentrations were determined by HPLC with a Sugar Pak column (Waters, Milford, USA) and a refractive index detector (Waters). Distilled water filtered by a MilliQ System (Millipore, USA) was used as the mobile phase at a flow rate of 0.4 ml min^{-1} at 90 °C. Ethanol was measured by GC (121 DFL Intersmat, Paris, France) using a 60/80 Carbopack B/5% Carbowax 20 M glass column (Supelco, Bellafonte, USA) and a flame ionization detector. The column temperature was set at 85 °C and the carrier gas was nitrogen.

RESULTS

Effect of individual hemicellulosic sugars on the induction of aldose reductase activity

The effect of individual sugars derived from hemicellulosic hydrolysates on the cell growth of *Candida guilliermondii* was first examined to determine fermentation parameters and major end products (Table 1). D-Glucose, D-mannose and D-galactose were rapidly and entirely consumed after 10, 14 and 38 h of growth, respectively. These sugars were utilized only for growth, ethanol and glycerol production (Table 1). Their corresponding polyols were not detected in the culture medium. The specific rates of D-xylose and L-arabinose consumption by cells were low, being 0.10 and 0.02 g g^{-1} h^{-1}, respectively. Complete consumption of xylose and arabinose was obtained after 117 and 181 h respectively. In contrast with the hexoses, *Candida guilliermondii* secreted xylitol from D-xylose and arabinitol from L-arabinose. The yeast cells accumulated 10.1 and 2.2 g L^{-1} of xylitol and arabinitol, respectively, at 92 and 110 h of growth (Table 1).

Cell growth on glycerol served as the control for the induction experiments and biomass production for the induction assay under resting cell conditions. Glycerol was consumed at a slower rate than the hemicellulosic sugars tested. Glycerol consumption was incomplete and reached 0.8 g L^{-1} after a culture time of 168 h (data not shown). The data obtained by screening cell extracts of *Candida guilliermondii*, grown in a variety of carbon sources, for NAD(P)H-dependent enzyme activities are shown in Table 2. The cell extracts possessed aldose reductase activity that was strictly dependent on the presence of NADPH as cofactor. No NADH-linked enzyme activity was found under the conditions of assay (data not shown). The highest activity of NADPH-linked aldose reductase was found in D-xylose-grown cells with L-arabinose as substrate. The NADPH-dependent aldose reductase activity in L-arabinose-grown cells was half of that in the D-xylose grown cell extract (Table 2). D-Xylose and L-arabinose were found to be effective inducers of aldose reductase activity. A low aldose reductase activity was observed in extracts of D-galactose and glycerol-grown cells, and even less in D-glucose or D-mannose-grown cells.

These aldose sugars also served as substrates for the reductase assay (Table 2). NADPH-linked aldose reductase activities with L-arabinose, D-galactose, D-glucose as substrates were observed in extracts of D-xylose and L-arabinose-grown cells. Low activities of NADPH-dependent aldose

TABLE 1

Fermentative parameters of *Candida guilliermondii* NRC 5578 grown on individual hemicellulosic sugars and glycerol

Carbon source (2%)	Parameters					Products ($g\ L^{-1}$)		
	T (h)	Substrate used (%)	μ_{max} (h^{-1})	Q_s ($g\ L^{-1}\ h^{-1}$)	q_s ($g\ g^{-1}\ h^{-1}$)	Polyol	Ethanol	Glycerol
D-Xylose	92	72	0.020	0.17	0.10	10.1*	0.06	0.9
L-Arabinose	110	52	0.017	0.11	0.02	2.2**	0	0
D-Galactose	38	100	0.031	0.47	0.45	0	5.4	0.5
D-Glucose	10	99	0.104	1.50	1.07	0	7.0	0.7
D-Mannose	14	99	0.065	1.10	1.15	0	6.5	1.1
Glycerol	48	1	0.011	0.005	0.005	0	0	Nd

*Xylitol.
**Arabinitol.
Nd = not determined.

TABLE 2

NADPH-dependent aldose reductase activities of *Candida guilliermondii* NRC 5578 grown in media containing hemicellulose-derived sugars and assayed with different aldoses as enzymatic substrates

Carbon source (2%) supporting growth[a]	NADPH-dependent aldose reductase activities ($mU\ mg^{-1}$ protein) Enzyme substrates				
	D-Xylose	L-Arabinose	D-Galactose	D-Glucose	D-Mannose
D-Xylose	1191	1525	540	127	42
L-Arabinose	614	839	270	90	30
D-Galactose	84	93	16	5	11
D-Glucose	14	14	4	0	0
D-Mannose	30	34	11	2	0
Glycerol	133	185	57	13	13

No NADH-dependent aldose reductase activity was found.
[a]Cells were grown on different carbon sources for the time during which the sugar concentration in the medium was 50% of the initial concentration.

reductase with D-xylose, L-arabinose, D-galactose, D-glucose, D-mannose as substrates were found in D-glucose, D-galactose and D-mannose-grown cell extracts. L-Arabinose was a 1.3 times more advantageous substrate than D-xylose for NADPH-linked aldose reductase activity, with the exception of the activities observed for the D-glucose-grown cell extract (Table 2).

Induction of aldose reductase activities during xylose fermentation

Studies of induction of aldose reductase activities were performed under growing and resting cell conditions. The addition of $20\ g\ L^{-1}$ D-xylose to the culture medium (Fig. 1(A)) induced the NADPH-dependent aldose reductase activity, which reached a maximal level after 50–72 h of growth and decreased steadily until 100 h of cell growth (Fig. 1(B)). This decrease can be accounted for by the cessation of the biosynthesis of the enzyme as a result of total xylose depletion, at 72 h. Another possible cause is the dilution of preexisting enzyme by 'de novo' protein synthesis due to cell growth. When cell growth approached the stationary phase, after 120 h (Fig. 1(A)), the aldose reductase activities became constant (Fig. 1(B)). The cell growth in the D-xylose medium showed the phenomenon of diauxic growth (Fig. 1(A)). After 86 h when all D-xylose was consumed, xylitol and ethanol produced from xylose in the medium were utilized for the production of biomass.

When the NADPH-dependent aldose reductase activity was assayed with L-arabinose as enzyme substrate (Fig. 1(B)), the evolution of aldose reductase activity with incubation time was similar to that obtained with D-xylose as substrate. However, the highest values were observed with L-arabinose as substrate.

The addition of 4% D-xylose to the incubation system with cells maintained under resting conditions, resulted in immedi-

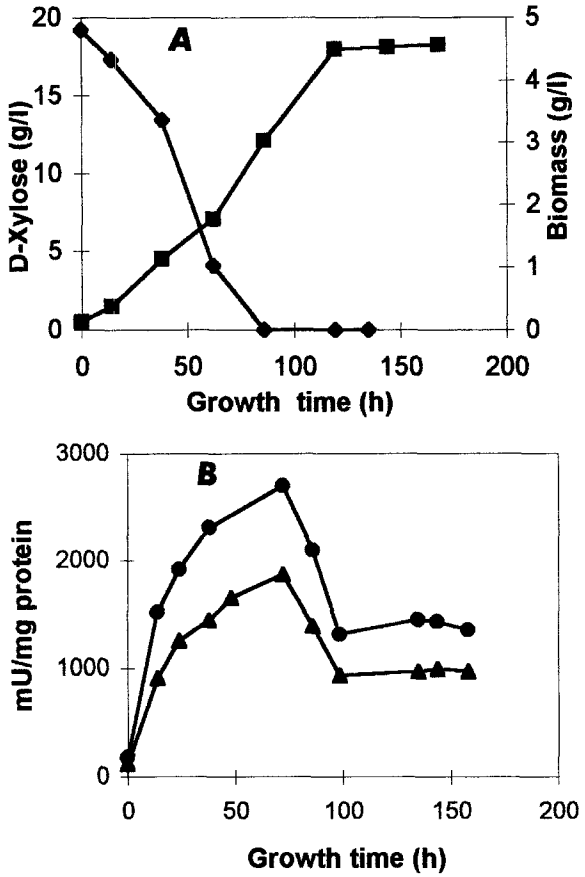

Fig. 1. Relationship between growth (A) and aldose reductase activity (B) by cells of *Candida guilliermondii* NRC 5578 as a function of time. Symbols: ◆: xylose; ■: biomass; aldose reductase activities with D-xylose (▲) and L-arabinose (●) as substrates of the enzymatic reaction.

ate induction of aldose reductase activities, which increased with time (Fig. 2). With D-xylose and L-arabinose as substrates, the induction of aldose reductase activity was increased maximally to three times that of their respective controls (Fig. 2). The level of induced aldose reductase activity began to decrease after 110 h, correlating with complete consumption of the inducer (data not shown). The evolution of induction of aldose reductase activities with time followed the same pattern but the level was half of that obtained from the cells under growing conditions.

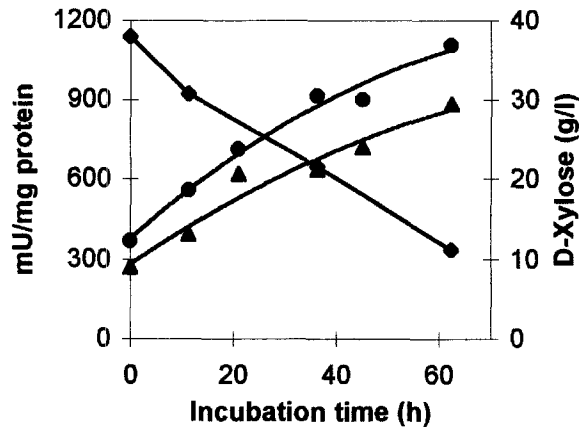

Fig. 2. Kinetics of induction of aldose reductase activity in resting cells of *Candida guilliermondii*. Symbols: ◆: xylose; aldose reductase activities with D-xylose (▲) and L-arabinose (●) as substrates of the enzymatic reaction.

Effect of initial inducer concentration on the induction of aldose reductase activity

The rate of aldose reductase activity by resting cells depends upon the initial inducer concentration employed (Fig. 3(A,B)). Addition of 9–80 g L^{-1} of D-xylose did not markedly increase the induced rate of synthesis of aldose reductase. However, when the inducer concentration was increased to the range of 125–350 g L^{-1}, marked differences were observed in their induction rate. At these latter concentrations, aldose reductase activity with D-xylose and L-arabinose as substrates decreased 2–4 times and 3–5.5 times, respectively at 46 h. With 270 and 350 g L^{-1} D-xylose the enzyme activities were

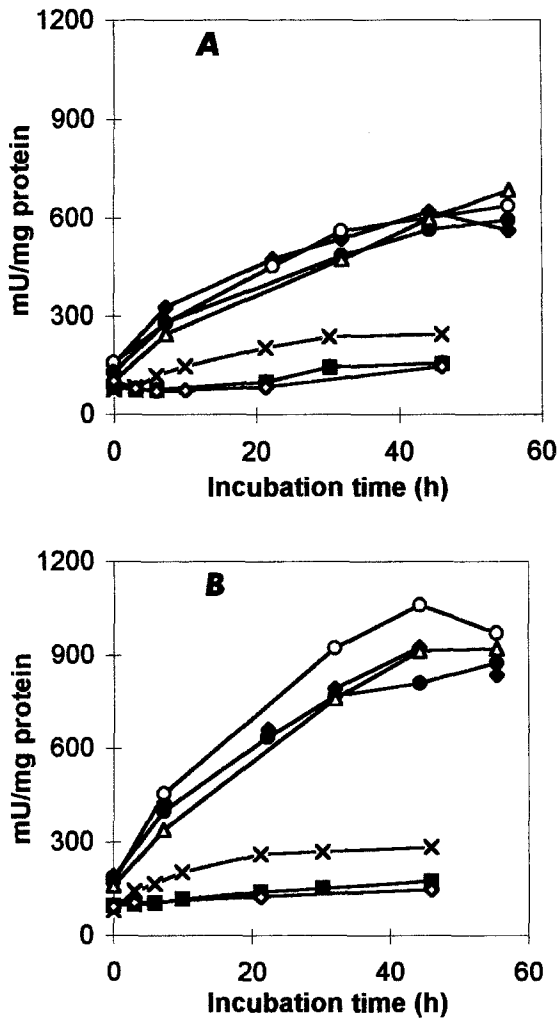

Fig. 3. Effect of initial D-xylose concentrations on the kinetics of induction of aldose reductase activity in *Candida guilliermondii* under resting cells conditions. Symbols: 9 (◆); 13 (○); 39 (●); 80 (△); 125 (×); 270 (■) and 350 g L^{-1} (◇). Aldose reductase activity with D-xylose (A) and L-arabinose (B) as substrates of the enzymatic reaction.

near the original levels. This result appears due to cell modification because of the high sugar concentrations and to a lysis of some cells. After 45–50 h induction, total depletion of 9 and 13 g L^{-1} D-xylose was observed (data not shown).

DISCUSSION

The growth of *Candida guilliermondii* under batch culture conditions using various sugars showed that the maximum growth rate was achieved on D-glucose followed by D-mannose and D-galactose. The other fermentation parameters with D-glucose and D-mannose as carbon sources were similar. All the hexoses tested were utilized by cells only for growth and ethanol production, and catabolism probably involved the Embden–Meyerhof–Parnas pathway, because their corresponding polyols were not detected in the culture medium. The specific growth rate on D-xylose and L-arabinose were five and six times, respectively, slower than for glucose. Production of xylitol and arabinitol from their pentoses as the major products, suggests that the metabolism of D-xylose and L-arabinose appears to be via a reduction and oxidation to D-xylulose, which is then phosphorylated to form D-xylulose-5-phosphate, an intermediate of the pentose phosphate pathway, like with other yeasts and fungi [23–25]. When D-xylose was completely depleted in the growth medium (at 86 h), xylitol produced from D-xylose was utilized by *Candida guilliermondii* as a carbon source for growth, leading to a progressive decrease in xylitol yield. This is in agreement with the results of Barbosa et al. [2].

The highest aldose reductase activity strictly dependent on NADPH from *Candida guilliermondii* was induced in D-xylose-grown cells followed by L-arabinose growth, with these inducing pentoses as effective substrates for the enzymatic reaction. This observation is in agreement with that found in *Pachysolen tannophilus* [5], but is in contradiction with other studies which showed better activity with D-xylose as substrate [25] or the simultaneous performance of inducing and enzymatic substrate pentose [17]. The very low level of aldose reductase activity observed in either D-galactose-, D-mannose- and D-glucose-grown cell extracts suggests that the mechanism leading to expression of this enzyme is under catabolite repression control, which is typically exerted by D-glucose or other rapidly metabolizable carbon sources [11,13,18]. This phenomenon was also observed in cells of *Aureobasidium pullulans* [17], *Pachysolen tannophilus* [3,5], and *Pichia stipitis* [3]. In contradiction to our results, a weak induction of aldose reductase activity by D-galactose was observed in some other yeast cells grown on D-galactose as sole substrate, or mixed sugars [3,5,17].

The aldose reductase activity induced in *Candida guilliermondii* by D-xylose and L-arabinose could be attributed to the presence of either a single aldose reductase with differing substrate specificities as observed in *Pachysolen tannophilus* [5] or multiple aldose reductases as found in *Pichia quercuum* [25] or *Aureobasidium pullulans* [17]. An experiment on the heat stability of the enzyme with D-xylose and L-arabinose as inducers showed that rates of heat inactivation of the aldose reductase were similar for both substrates of enzymatic reactions and independent of the temperatures at which the enzyme was preincubated. These results suggest that the aldose reductase activity in *Candida guilliermondii* may result from a single NADPH-dependent enzyme with differing substrate specificities.

The aldose reductase activity induced by D-xylose was dependent on the growth process. The induced enzyme level was twice as high in growing cells as in resting cells. With resting cells, the oxygen supply to cells during the induction process slows down as a result of the higher cell densities in the induction system. The correlation between oxygen supply and the level of NAD(P)H aldose reductase activity has been reported by some authors [1,21]. The first consequence of oxygen limitation may be an imbalance in cofactor production, i.e. the intracellular NADPH pool may decrease and cause a reduction in the level of induction of aldose reductase in *Candida guilliermondii* since protein synthesis requires NADPH [12]. The second consequence of oxygen limitation may be that the ATP yield is not sufficient for all cell activities. Thus, uptake of D-xylose into the cells limits the rate of aldose reductase induction by *Candida guilliermondii* as a result of the decrease of intracellular inducer concentration, because the sugar transport system in the xylose fermenting yeasts is usually energy dependent [15,16].

The experiment on the effect of the initial inducer concentration on the kinetics of aldose reductase induction showed that D-xylose concentrations at which the maximum enzyme induction was observed, occurred with 9–80 g L^{-1}. Significant differences in enzyme level were not observed in this range, neither with D-xylose nor with L-arabinose as the substrate for the enzymatic reaction. However, in the range of 125–350 g L^{-1} of D-xylose, aldose reductase activity did not markedly increase over that obtained with the original level, showing that the induction rate of aldose reductase was not influenced by high initial sugar concentrations. Discrepancies in the effect of initial xylose concentration on xylose reductase activity of the same strain of *Candida guilliermondii* was observed by Nolleau et al. [21]. They reported that under aerobiosis the maximal xylose reductase activity was obtained with 200–300 g L^{-1} of D-xylose. This discrepancy can possibly be attributed to differences in growth conditions, particularly oxygen supply. Enzyme induction studies on mixed hemicellulosic sugars are currently under investigation to determine whether the performance of D-xylose utilization in *Candida guilliermondii* is related to repression of aldose reductase activity.

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NOMENCLATURE

Q_s (g L^{-1} h^{-1}) average volumetric uptake rate of carbon substrate

q_s (g g^{-1} h^{-1})	average specific uptake rate of carbon substrate
μ_{max} (h^{-1})	maximum specific growth rate
T (h)	culture time at which fermentation parameters are calculated

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