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Chemostat study of xylitol production by *Candida guilliermondii*

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Abstract The mechanism of production of xylitol from xylose by *Candida guilliermondii* was studied using chemostat cultures and enzymatic assays. The maximum dilution rate in aerobic conditions was 0.34 1/h. No xylitol was produced. Under oxygen-limited conditions xylose uptake was impaired and glycerol accumulated but no xylitol was detected. Under transient oxygen limitation, caused by a gradual decrease in the agitation rate, onset of xylitol, acetate and residual xylose accumulation occurred simultaneously when q_{O2} dropped below 25 mmol/C-mmol cell dry weight (CDW) per hour. Ethanol and glycerol started to accumulate when q_{O2} dropped below 20 mmol/C-mmol CDW per hour. The highest in vitro enzyme activities were found at the lowest dilution rate studied (0.091/h) under aerobic conditions. The amount of active enzymes or cofactor availability did not limit the rate of xylose consumption. Our results confirm that a surplus of NADH during transient oxygen limitation inhibited the activity of xylitol dehydrogenase which resulted in xylitol accumulation. Phosphoglucoisomerase (E.C. 5.3.1.9.) and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) activities suggest re-shuttling of the metabolites into the pentose phosphate pathway.

Introduction

Considerable efforts have been focused on microbial reduction of D-xylose to xylitol as an alternative to chemical catalysis. In competition with the chemical process the volumetric productivity of a bioprocess is one of the critical factors, having a direct impact on both variable and fixed cost elements (Wilke 1999). Wilke presents cost estimates for bulk products manufactured by bioprocesses based on the following values: (1) 150 g/l product concentration, (2) 80% w/w yield on carbohydrate feedstock and (3) a feedstock price of US$ 200/ton. Consideration of environmental issues and the use of crude industrial sidestreams as a raw material may improve the economics of a bioprocess. To improve volumetric productivity, it is important to understand how to control and channel the cell metabolism towards a desired product. This approach is particularly attractive for those metabolic pathways which consist of only a few enzymatic reactions, such as the reduction of xylose to xylitol. As an experimental model xylose reduction offers possibilities for studying metabolic regulation and applying metabolic flux analysis and metabolic control analysis to formulate cell metabolism mathematically.

The conversion of xylose to xylitol by yeasts is thoroughly described in the literature (Smiley and Bolen 1982; Lachke and Jeffries 1986; Slininger et al. 1987; Prior et al. 1989; Hahn-Hägerdahl et al. 1994). Various process variables such as inocula, substrate, media, temperature, pH and aeration have an impact on xylitol production, and one of the most critical is oxygen availability. Xylitol formation is favoured under oxygen-limited conditions, because NADH accumulates and inhibits NAD-linked xylitol dehydrogenase (Winkelhausen and Kuzmanova 1998). According to Kastner et al. (1999), *Candida shehatae* does not grow anaerobically on D-glucose or D-xylose. When anaerobic conditions were imposed, cell viability declined nine times faster in D-xylose fermentation than in D-glucose fermentation. A step change from aerobic to anaerobic conditions resulted in ethanol production from D-glucose and ethanol and xylitol production from D-xylose.

Roberto et al. (1999) studied the effect of $k_L a$ on the conversion of xylose to xylitol by *Candida guilliermondii* (FTI 20073) in a batch fermentation. With rice straw

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hydrolysate as a substrate (containing 62 g/l xylose) the maximum volumetric productivity was 0.52 g/l per hour and the highest xylitol concentration (36.8 g/l) was attained at $k_L a$ 15 1/h after 70 h cultivation. Domínguez et al. (1999) studied xylitol production by Ca-alginate entrapped *Debaryomyces hansenii* and *C. guilliermondii*. They reached volumetric productivities of 0.58 and 0.91 g/l per hour respectively. Barbosa et al. (1988) achieved a 77.2 g/l xylitol yield from 104 g/l xylose with *C. guilliermondii* FTI 20037 using aerobic high-cell-density culture and defined medium. Meyrial et al. (1991) achieved a 221 g/l xylitol yield from 300 g/l xylose with an average specific production rate of 0.19 g/g CDW per hour. Ojamo (1994) demonstrated that *C. guilliermondii* VTT-C-71006 is an efficient xylitol producer. A xylitol yield of 0.74 g/g xylose was obtained within 50 h at an initial xylose concentration of 100 g/l.

The first two enzymes in the xylose metabolising pathway are xylose reductase and xylitol dehydrogenase (Verduyn et al. 1985; Rizzi et al. 1988). The thermodynamic equilibrium of both reactions favours the formation of xylitol (Rizzi et al. 1988). In *C. shehatae* aerobic xylose consumption is possibly transport-limited (Alexander et al. 1988) or affected by the levels of xylose reductase or glucose-6-phosphate dehydrogenase (the source of NADPH), whereas in anaerobic conditions the NADH-linked xylose reductase is probably rate-limiting (Alexander et al. 1988). According to Bruinenberg et al. (1983), both the hexose monophosphate pathway and NADP-dependent isocitrate dehydrogenase are potential sources of NADPH during growth of *Candida utilis* on glucose, xylose and gluconate. According to Verduyn et al. (1985), xylose reductase from *Pichia stipitis* was active with both NADH and NADPH, but the ratio of activity varied with the concentration of coenzymes. Neuhauser et al. (1997) concluded that aldose reductase (ALR) from *Candida tenuis* prefers NADPH, owing to better apparent binding of the phosphorylated form of the coenzyme.

Our study aims at elucidating the mechanism of xylitol production in *C. guilliermondii* using a chemostat culture as a study method. The enzyme activities analysed in vitro were chosen to study the involvement of glycolysis and the pentose phosphate pathway in xylose metabolism by *C. guilliermondii*. Maleate dehydrogenase was taken as a marker enzyme for tricarboxylic acid (TCA) cycle activity. A novel experimental set-up was employed in this work: transient oxygen limitation was created in the chemostat by decreasing the agitation gradually. The effect of transient oxygen limitation was compared to that of constant oxygen limitation.

Materials and methods

Organism, maintenance and inoculum preparation

Candida guilliermondii VTT-C-71006 was obtained from VTT Biotechnology and Food Research Laboratory (Espoo, Finland). Frozen stock cultures containing 20% (w/v) glycerol were stored in 2-ml ampoules at −70 °C. Inoculum for fermentation was prepared in 250-ml shake flasks grown overnight on YM medium at 30 °C and 200 rpm.

Shake flask and chemostat experiments

YM medium contained 3 g/l yeast extract (Difco), 3 g/l malt extract (Difco), 5 g/l bactopeptone (Difco) and 10 g/l glucose (Fluka). The 250-ml shake flasks were incubated in a shaker at 30 °C and 220 rpm with 100 ml YM medium. Mineral medium was prepared according to Verduyn et al. (1992) and contained per litre: $(NH_4)_2SO_4$ 5.0 g, KH_2PO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, EDTA 15.0 mg, $ZnSO_4 \cdot 7H_2O$ 4.5 mg, $CoCl \cdot 6H_2O$ 0.3 mg, $MnCl_2 \cdot 4H_2O$ 1.0 mg, $CuSO_4 \cdot 5H_2O$ 0.3 mg, $CaCl_2 \cdot 2H_2O$ 4.5 mg, $FeSO_4 \cdot 7H_2O$ 3.0 mg, $NaMoO_4 \cdot 2H_2O$ 0.4 mg, H_3BO_4 1.0 mg, KI 0.1 mg and silicon-based antifoam agent 0.05 mg (BDH). Mineral medium was autoclaved for 20 min at 120 °C. After autoclaving a filter-sterilised vitamin solution was added to give a final concentration per litre of biotin 0.05 mg, calcium pantothenate 1.0 mg, nicotinic acid 1.0 mg, *myo*-inositol 25 mg, pyridoxal hydrochloride 1.0 mg and *para*-aminobenzoic acid 0.2 mg. Glucose and xylose were sterilised at 110 °C (to minimise the sugar degradation) for 20 min and added separately to the growth medium in order to give a final concentration of 250 C-mM. Culture purity was monitored on a regular basis by phase contrast microscopy.

Chemostat cultivations were carried out in a 2-l fermenter (Braun MD) on a mineral medium at 30 °C with a stirrer speed of 600 rpm. The culture pH was set at 5.0 and the dilution rate was adjusted to a desired value. The working volume of 1000 ml was kept constant by removing the effluent with a peristaltic pump (Watson-Marlow 505 U) connected to a PID (proportional, integral, derivative)-controlled load cell. The actual working volume was determined at the end of each experiment. The culture pH was kept constant by addition of 2 M KOH. The airflow rate was set to maintain the dissolved oxygen concentration above 30% in all aerobic cultivations and was controlled using a massflow controller (oxygen-limited cultivations excluded) (Bronkhurst HiTec, Ruurlo, Holland). Oxygen-limited conditions or non-oxygen-limited conditions were consequently verified with measuring data from a mass spectrometer (VG-Prima 600). The dissolved oxygen concentration was measured with an oxygen electrode (Ingold). Oxygen limitation (constant) was established by mixing air with nitrogen gas in 1:5 ratio. Transient oxygen-limited conditions were created by a profile where agitation speed decreased gradually by 50 rpm per hour to a minimum value of 100 rpm.

Exhaust gas analysis

The fermentation exhaust gas was cooled to 4 °C in a condenser to prevent the evaporation of volatile compounds before it entered the mass spectrometer. Carbon dioxide, oxygen, argon and nitrogen were analysed from the exhaust gas. In calculating the oxygen consumption rates and carbon dioxide production rates a temperature of 30 °C was assumed, and the air pressure measurement was taken from the daily weather forecast.

Cell dry weight measurements

Culture samples (10 ml) were vacuum-filtered through pre-weighed nitrocellulose filters (0.45 μm, Schleicher & Schuell), washed with Milli-Q water and then dried in a microwave oven for 20 min (Ignis, Japan). The standard deviation of this technique was determined to be less than 1%. The total organic carbon of lyophilised biomass was analysed by a Shimadzu 5000 (Japan) carbon analyser. The carbon content of analysed samples varied between 0.39% and 0.42%.

Substrate and metabolite analysis

Sample volumes of 1.5 ml collected from the chemostat cultures were centrifuged at 10,000 rpm for 5 min (Heraeus Sepatech, Biofuge A, Germany) and the supernatant was stored at −20 °C for further analysis. Xylose, glucose, xylitol, glycerol, acetate and ethanol concentrations were analysed by high-performance liquid chromatography (HPLC). The set-up included an HPX-87H Aminex ion exclusion column (Bio-Rad) and two detectors in series, a Waters 410 refractive index and a Waters 486 UV detector. The column was maintained at 60 °C and eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min.

Preparation of cell-free extract

Cell-free extract was prepared according to De Jong-Gubbels et al. (1996) with the exception that cells were disrupted with a Vibra Cell sonicator (VCX600, USA). Ten 30-s pulses were applied with a 30-s cooling period between each pulse.

Enzyme assays

Enzyme assays were performed at 30 °C with freshly prepared cell free extracts using a Shimadzu UV-2100 spectrophotometer. Reaction rates, corrected for endogenous rates, were proportional to the amount of extracts added. Xylose reductase (E.C. 1.1.1.21) and xylitol dehydrogenase (E.C. 1.1.1.9) were assayed as described by Verduyn et al. (1985). Pyruvate decarboxylase (E.C. 4.1.1.1), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and alcohol dehydrogenase (E.C. 1.1.1.1.) were assayed as described by Postma et al. (1989). Maleate dehydrogenase (E.C. 1.1.1.37), phosphoglucoisomerase (E.C. 5.3.1.9.), NAD-dependent isocitrate dehydrogenase (E.C. 1.1.1.41) and NADP-dependent isocitrate dehydrogenase (E.C. 1.1.1.42) were assayed according to Bergmeyer (1984). One unit of activity was defined as the amount of enzyme catalysing the conversion of 1 μmol substrate per minute. Specific activities are expressed as units per mg protein.

Protein determination

The protein content of whole cells was assayed by a modified biuret method (Verduyn et al. 1990) and with the Bio-Rad protein assay kit. The protein concentration in cell-free extracts was determined by the Lowry method using bovine serum albumin (Sigma) as a standard.

Results

In a shake-flask cultivation, when 2.97 C-mol/l xylose was used as the sole carbon substrate, the xylitol yield from xylose ($Y_{xol/xyl}$) was 0.61 C-mol xylitol/C-mol xylose and the biomass yield from xylose ($Y_{x/xyl}$) was 0.12 C-mol CDW/C-mol xylose (Fig. 1). Xylose consumption by *C. guilliermondii* was repressed by glucose on a complex medium in shake flasks (results not shown).

Overview of the chemostat experiments is presented in Table 1. The maximum specific growth rate of *C. guilliermondii* with xylose was determined (Fig. 2). Biomass production decreased and residual xylose accumulated strongly above the dilution rate of 0.35 1/h. The highest specific xylose uptake (q_{xyl}) was 0.66 C-mol xylose/C-mol CDW per hour at a 0.387/h dilution rate. The highest yield on xylose ($Y_{x/xyl}$) was 0.58 C-mol CDW/C-mol xylose at dilution rates of 0.273 and

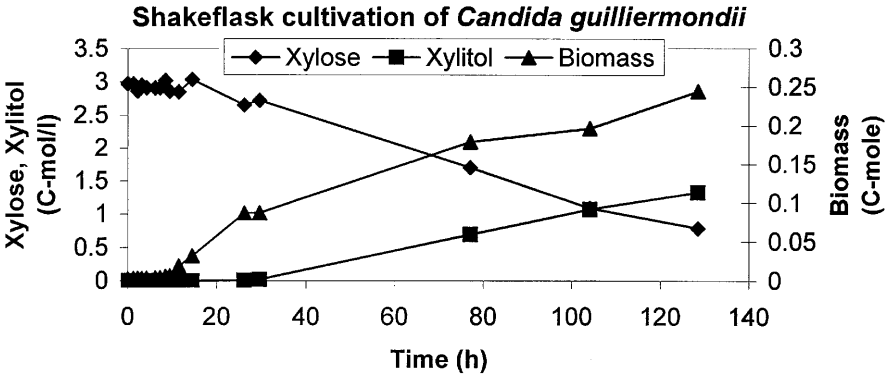

Fig. 1 Shake-flask cultivation of *Candida guilliermondii* on YM medium. Cell dry weight was analysed in the end of the experiment and biomass values were calculated accordingly from OD_{600} measurements

Table 1 Overview of chemostat culture data of *Candida guilliermondii*. Cultivations were done on a mineral medium at 30 °C at pH 5. Oxygen-limited cultivation was done by mixing air and nitrogen gas in a ratio of 1:5. In order to establish a steady state at least five volume changes were allowed before sampling

Dilution rate (1/h)	Cell dry weight (C-mol/l)	C_{xyl} (C-mol/l, feed)	C_{xyl} (C-mol/l, residual)	C_{gly} (C-mol/l, residual)	$Y_{x/xyl}$ (C-mol CDW/ C-mol xylose)	q_{O2} (mol/C-mol CDW per hour)	q_{CO2} (C-mol/C-mol CDW per hour)	C balance %
0.091	0.116	0.218	0.00	0.00	0.53	0.83	0.87	99.3
0.186	0.130	0.239	0.00	0.00	0.54	0.68	0.72	93.4
0.273	0.140	0.243	0.001	0.00	0.58	0.61	0.66	96.2
0.387	0.132	0.242	0.015	0.00	0.58	0.63	0.67	97.3
0.097[a]	0.059	0.238	0.100	0.33	0.43	0.22	0.96	91.2

[a] Oxygen-limited chemostat

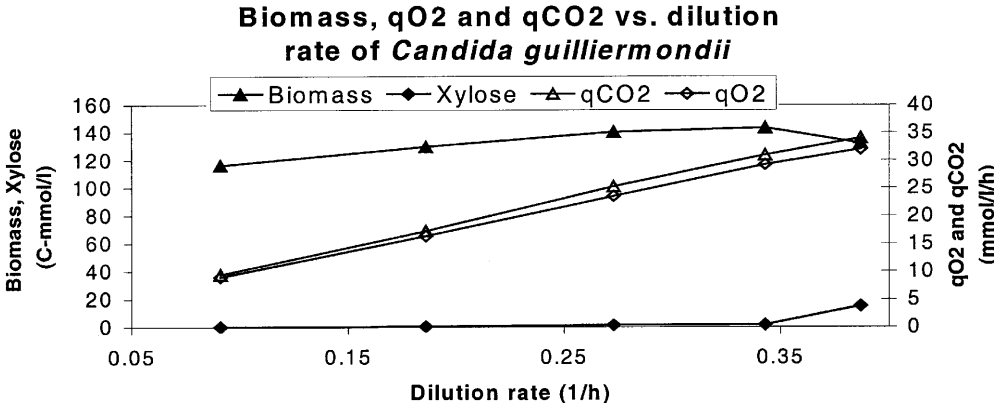

Fig. 2 Biomass, q_{O2} and q_{CO2} vs dilution rate of *C. guilliermondii* on a mineral medium. Steady state was established after five volume changes. Cultivation pH was set to 5.0 and temperature 30 °C. No other metabolite was produced than biomass and carbon dioxide. The maximum dilution rate was determined at the point where xylose starts to accumulate in the fermenter

0.387 1/h (Table 1). The specific oxygen consumption (q_{O2}) and carbon dioxide production (q_{CO2}) rates were highest at a dilution rate of 0.091/h and lowest at dilution rates of 0.273 1/h and 0.387 1/h, respectively, in fully aerobic conditions. No other metabolites than biomass and carbon dioxide were produced in these conditions.

When *C. guilliermondii* was grown under oxygen limitation (constant), only glycerol was produced in addition to biomass and carbon dioxide at the dilution rate of 0.097 1/h (Table 1). The specific oxygen consumption and carbon dioxide production rates were 0.22 mol/C-mol CDW per hour and 0.96 C-mol/C-mol CDW per hour respectively. The residual xylose concentration under oxygen-limited conditions was 0.10 C-mol/l (Table 1). No xylitol was produced in these conditions. Constant oxygen limitation impaired xylose consumption. Under these conditions the maximum specific uptake of xylose (q_{xyl}) was 0.23 C-mol/C-mol CDW per hour, compared to 0.66 C-mol/C-mol CDW per hour in fully aerobic conditions.

In order to understand why xylitol accumulated in shake-flask cultures but not in steady-state chemostat cultures, transient oxygen limitation by a gradual reduction in agitation speed (50 rpm/h) was established (Fig. 3). At a dilution rate of 0.048 1/h with 3.81 C-mol/l xylose as carbon substrate, the onset of xylitol accumulation (23.8 C-mmol/l) and residual xylose accumulation (2.58 C-mmol/l) occurred when q_{O2} dropped below 24.71 mmol/C-mmol CDW per hour. After 9 h q_{O2} had reached the value of 4.10 mmol/C-mmol CDW per hour and the concentrations of xylitol and residual xylose were 201.6 and 270.6 C-mmol/l, respectively. When the experiment was continued for another 27 h with a constant q_{O2} value of 3.53 mmol/C-mmol CDW per hour, the residual xylose accumulation increased to 1.59 C-mol/l concomitantly with 290.5 C-mmol/l xylitol accumulation (results not shown). At dilution rates of 0.012 1/h and 0.027 1/h, with 3.67 C-mol/l xylose as a substrate, the onset of xylose and xylitol accumulation occurred at q_{O2} values of 10.18 and 9.86 mmol/C-mmol CDW per hour, respectively. The corresponding xylitol

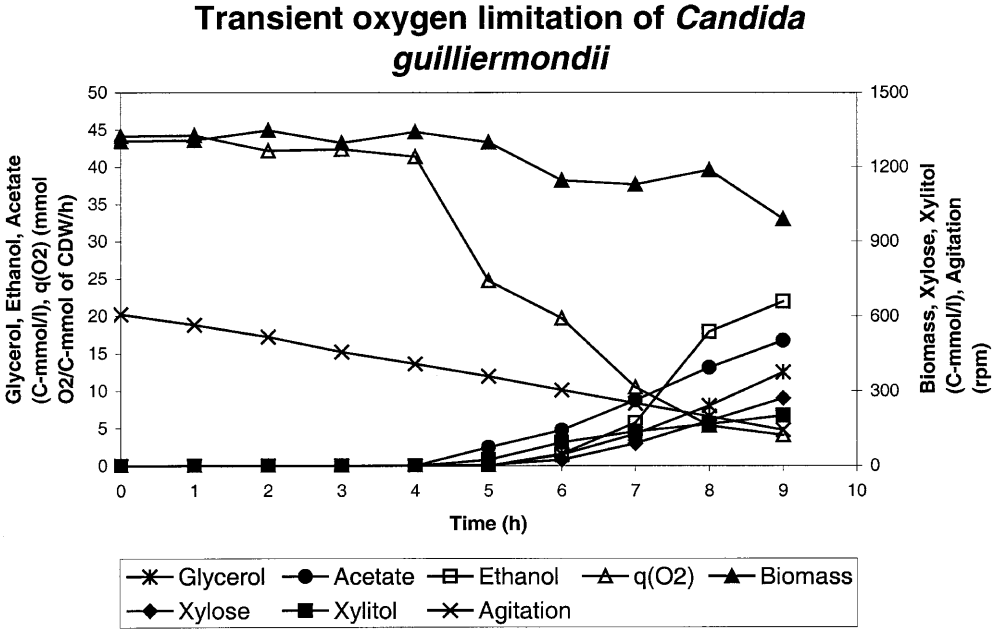

Fig. 3 Transient oxygen limitation of *C. guilliermondii* at the dilution rate of 0.048 1/h with xylose (3.81 C-mol) as a substrate. In order to establish a steady state at least five volume changes were allowed before sampling. Agitation was decreased gradually by 50 rpm/h. As a consequence of redox imbalance, glycerol, ethanol and acetate are produced together with xylitol. Xylose uptake was impaired in these conditions

concentrations were 13.5 and 23.15 C-mmol/l respectively (results not shown).

Results from enzyme assays are shown in Table 2. The enzyme assays showed that activities of xylose reductase and xylitol dehydrogenase decreased with increasing xylose consumption rate (Table 2). The same was also seen with phosphoglucoisomerase, maleate dehydrogenase, NADP-dependent isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase (Table 2). The only measured enzyme activity that clearly increased with increasing dilution rate was NAD-dependent isocitrate dehydrogenase, except for the decline at the dilution rate of 0.186 1/h. Under oxygen limitation (constant) at the dilution rate 0.097 1/h the xylose reductase and xylitol dehydrogenase activities were only 7.5% and 34%, respectively, of those under fully aerobic conditions at a similar dilution rate (Table 2). The activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were 71% and 35%, respectively, of those in fully aerobic conditions. Despite the oxygen-limited conditions, alcohol dehydrogenase and pyruvate decarboxylase activities were not significantly elevated (results not shown). The maleate dehydrogenase activity was at the same level in oxygen-limited conditions at the dilution rate of 0.097 1/h and at the highest dilution rate, 0.387 1/h, under aerobic conditions (Table 2).

Discussion

Under aerobic conditions *C. guilliermondii* did not produce xylitol in chemostat culture even after the onset of xylose accumulation and biomass reduction. Limited respiratory capacity cannot explain the xylitol production mechanism in *C. guilliermondii* as it explains ethanol production from glucose in the long-term Crabtree effect in fully aerobic conditions in yeasts (Fiechter et al. 1981). The xylose reductase activity at the dilution rate of 0.097 l/h under oxygen limitation was similar to that at the dilution rate of 0.387 1/h in fully aerobic conditions. In the former case the rate of xylose consumption was 0.23 C-mol/C-mol CDW per hour and in the latter case 0.67 C-mol/C-mol CDW per hour. The intracellular amount of NADPH-dependent xylose reductase protein is probably not rate-limiting in xylose consumption under constant oxygen-limited conditions. This indicates that oxygen limitation impairs xylose uptake. It may have a direct effect on xylose permease or surplus of NADH may act as an inhibitor for strictly NADPH-dependent xylose reductase. Furthermore, the decreased ATP generation may inhibit the xylulokinase activity, which might decrease the xylose consumption rate. The conflict between increased xylose consumption rate and decreased enzyme activities was also seen in fully aerobic conditions (Table 2). At the highest dilution rate (0.387 1/h) the IDP activity increased slightly. The maleate dehydrogenase activity had approximately the same value under oxygen limitation (dilution rate = 0.097 1/h) and at the highest dilution rate in fully aerobic conditions. Assuming that this activity is a marker enzyme for TCA cycle function, oxygen limitation and a high dilution rate had somewhat comparable physiological effects. This suggests that a surplus of NADH generated at the highest dilution rate inhibited the pyruvate dehydrogenase activity, which resulted in decreased maleate dehydrogenase activity. In *Saccharomyces cerevisiae* NADH exhibits competitive inhibition to pyruvate dehydrogenase with K_i of 23 μmM (Pronk et al. 1996).

Under constant oxygen limitation with glucose as the carbon source, ethanol, acetate and glycerol were produced and glucose was completely consumed (data not shown). According to Winkelhausen and Kuzmanova (1998) oxygen limitation inhibits xylitol dehydrogenase activity through NADH accumulation, which results in xylitol accumulation. However, constant oxygen limitation did not result in xylitol accumulation in *C. guilliermondii*. Oxygen limitation impaired xylose consumption and produced a minute amount of glycerol. This seems to be the way in which *C. guilliermondii* deals with redox imbalance, similarly to *S. cerevisiae*, where glycerol production acts as a redox sink for excess NADH (Oura 1977). When transient oxygen limitation was applied, xylose consumption was impaired and resulted in production of xylitol, acetate, glycerol and ethanol. The difference in metabolite production from xylose under constant versus transient oxygen limitation indicates that the degree of inhibition of glycolytic pathway enzymes is dependent on the intracellular NADH concentration in *C. guilliermondii*. Moreover,

Table 2 Comparison of enzyme activities of *C. guilliermondii* in the chemostat with xylose as the carbon substrate. Activities are expressed as U/mg protein and results are given as mean ± standard deviation. All measurements were performed in duplicate

Dilution rate (1/h)	Xylose reductase NADPH	Xylitol dehydrogenase NAD	Phosphogluco-isomerase	Maleate dehydrogenase NADH	NAD-dependent isocitrate dehydrogenase NAD	NADP-dependent isocitrate dehydrogenase NADP	Glucose-6-phosphate dehydrogenase
0.091	0.80 ± 0.14	2.31 ± 0.05	0.38 ± 0.03	20.02 ± 0.16	0.17 ± 0.00	0.20 ± 0.01	0.74 ± 0.29
0.186	0.26 ± 0.11	0.94 ± 0.21	0.25 ± 0.04	12.15 ± 1.15	0.01 ± 0.01	0.19 ± 0.01	0.49 ± 0.01
0.273	0.03 ± 0.00	0.4 ± 0.02	0.08 ± 0.01	4.50 ± 1.50	0.27 ± 0.04	0.06 ± 0.02	0.08 ± 0.01
0.387	0.07 ± 0.01	0.65 ± 0.12	0.10 ± 0.03	8.57 ± 0.04	0.34 ± 0.06	0.05 ± 0.00	0.19 ± 0.05
0.097[a]	0.06 ± 0.03	0.79 ± 0.25	0.27 ± 0.03	7.18 ± 0.14	0.09 ± 0.04	0.10 ± 0.06	0.26 ± 0.08

[a] Oxygen-limited chemostat

the inhibition may happen sequentially due to a steady increase in intracellular NADH accumulation. This sequence may have a crucial role in determining the fate of a substrate, such as uptake, fermentation or oxidation. However, this view may oversimplify the complexity of metabolic network under these transient conditions.

According to Handumrongkul et al. (1998), xylose reductases of *C. guilliermondii* and *Pichia stipitis* contain identical numbers of cysteine residues in identical locations, but in *C. guilliermondii* xylose reductase there is an additional histidine residue. This may contribute to the preference of NADPH as a cofactor for the *C. guilliermondii* enzyme. According to Bohren et al. (1991), lysine residue has a crucial role in cofactor binding in human aldose reductase. The lysine residue binds with the pyrophosphate bridge of NADPH (Wilson et al. 1992). Inactivation of yeast aldose reductase results from the lysine modification in or at least near the adenosine portion of the NAD(P)H-binding site (Neuhauser et al. 1997). It is possible that increased NADH accumulation concomitant with decreased activities of NADPH-generating enzymes may affect the active centre and its cofactor preference, particularly under in vivo conditions. This would explain the varying cofactor dependence of some enzymes due to the cultivation conditions. However, in the case of *C.guilliermondii* NADH cannot be used as a cofactor for xylitol production effectively. Small NADH-dependent activities of xylose reductase have been measured (Nolleau et al. 1993). We were not able to measure any NADH-dependent activity for xylose reductase in vitro (results not shown).

The availability of cofactors may be considered as one candidate for a rate-limiting step in xylose conversion to xylitol in a recombinant *S. cerevisiae* in anoxic conditions (Hallborn et al. 1994). In fully aerobic conditions the decreasing NADPH-dependent xylose reductase activity with an increased specific xylose uptake rate does not support this hypothesis in *C. guilliermondii*. The expression of NAD- and NADP-dependent isocitrate dehydrogenase varies with increasing dilution rate. Unfortunately, in vitro enzyme assays cannot take into account the localisation of the different isoenzymes of isocitrate dehydrogenase and thus their expression due to the varying conditions. However, the decreasing activity of NADP-dependent isocitrate dehydrogenase with increasing xylose consumption rate suggests that this enzyme cannot be the only source of NADPH for xylose reductase in these conditions. With the xylose reductase of *Pachysolen tannophilus* the varying cofactor dependence was found to be due to the aeration conditions (Winkelhausen and Kuzmanova 1998). The activities of glucose-6-phosphate dehydrogenase and phosphoglucoisomerase measured when xylose is a substrate may be an indication that metabolites are recirculated through the pentose phosphate pathway in order to regenerate cofactors.

In this work we have shown that it is possible to direct most of the xylose into xylitol by *C. guilliermondii*. Only a small part of xylose goes into biomass under varying oxygenation levels in shake-flask cultures. We have also elucidated the xylitol production mechanism of *C. guilliermondii* through detailed chemostat experiments and by measuring in vitro enzyme activities. Xylitol production is not growth-related but a consequence of redox imbalance. The enzyme activities assayed together with the quantitative data from chemostat cultivations support the conclusion that oxygen limitation impairs xylose uptake. In addition, recirculation of metabolites into the pentose phosphate pathway is suggested. The extent of active protein or cofactor availability is probably not rate-limiting in xylose consumption. The steady increase in intracellular NADH concentration may inhibit the glycolytic pathway enzymes in a chain. The order of inhibition may have a determining role in the fate of a substrate, e.g. oxidation, fermentation or inhibition. Finally, we have developed a novel transient experimental technique which can be extended to all growth parameters. This approach opens up new possibilities in studying in vivo metabolic regulation under controlled conditions that imitate batch fermentation. We may conclude that by selecting an appropriate cultivation method, adjusting oxygen availability and recirculating xylose substrate, *C. guilliermondii* could be applied in the industrial production of xylitol from xylose.

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