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 l/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 $q_{O_2}$ dropped below 25 mmol/C-mmol cell dry weight (CDW) per hour. Ethanol and glycerol started to accumulate when $q_{O_2}$ 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; Slinger 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...
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 kₜₐ at 15 l/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 xylitol 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 supposedly 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 glutonate. 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 bactopeptide (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: (NH₄)₂SO₄ 5.0 g, KH₂PO₄ 3.0 g, MgSO₄·7H₂O 0.5 g, EDTA 15.0 mg, ZnSO₄·7H₂O 4.5 mg, CoCl₂·6H₂O 0.3 mg, MnCl₂·4H₂O 1.0 mg, CuSO₄·5H₂O 0.3 mg, CaCl₂·2H₂O 4.5 mg, FeSO₄·7H₂O 3.0 mg, NaMoO₄·2H₂O 0.4 mg, H₂BO₃ 1.0 mg, KI 0.1 mg and silicon-based anti-foam 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) (Bronkhorst 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-weighted nitrocellulose filters (0.45 μm, Schleicher & Schuell), washed with Milli-Q water and then dried in a microwave oven for 20 min (Igins, 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%.