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BIOTECHNOLOGICAL PRODUCTION OF XYLITOL WITH *CANDIDA* YEASTS

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ABSTRACT

The aim of this study was to develop a biotechnological production process for xylitol. The xylitol production characteristics of *Candida millerii*, *Candida guilliermondii* and *Candida tropicalis* were compared. *C. tropicalis* was the best xylitol producer. A volumetric productivity of 5.7 g xylitol L^{-1} h^{-1} was achieved with 69 % yield from D-xylose on a mineral medium with a modified repeated fed batch production method. The xylitol production mechanism was confirmed by chemostat cultivation studies. Xylitol production is a consequence of an accumulation of intracellular NADH under oxygen limitation. NADH inhibits xylitol dehydrogenase enzyme (XDH) resulting in xylitol excretion out of the cell. The intracellular metabolic fluxes were studied by *in vitro* enzyme assays and more theoretically by Metabolic Flux Analysis (MFA). The determined enzyme activities revealed that different metabolic pathways are operating simultaneously enabling substrate cycling to maintain ATP turnover and redox balance under oxygen limited conditions. It was possible to calculate the ATP and NAD(P)H turnover by MFA. Connection between the cofactor dependency of xylose reductase (XR) and redox metabolite production was illustrated by a novel experimental set-up using an ascending xylose feed rate and descending oxygen consumption rate profiles simultaneously. This indicated that *C. tropicalis,* which XR is dual dependent is more responsive in respect to xylitol production under oxygen limited conditions than *C. guilliermondii*, which XR is only NADPH dependent. The role of additional NADH on xylitol flux was further confirmed by formate feeding. Formate is catabolised to NADH and CO_2 in the cell. *C. tropicalis* resulted in increased xylose uptake and xylitol production compared to *C. guilliermondii.* XR was purified from *C.guilliermondii* and its characteristics was studied. *C. milleri* was able to produce minor amounts of xylitol from xylose in the presence of glucose.

PREFACE

This work was carried out during the years 1998 – 2001 at the Laboratory of Bioprocess Engineering at the Department of Chemical Techology at Helsinki University of Technology. I would like to thank professor Leisola, the coordinator of this project, for being himself and taking care of the creative and open-minded atmosphere in our laboratory. Also, my thanks are due to my supervisors Ph.D. Aristos Aristidou, for his expertise and Ph.D. Heikki Ojamo, for his long time friendship and pointing me the way to the world of chemostats.

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Olen hyvin kiitollinen vanhemmilleni, Seijalle ja Göstalle, siskolleni Pirjolle ja veljelleni Kaijulle ja heidän perheillensä heidän pyyteettömästä tuestaan. Osoitan kiitokseni myös parhaimmalle ystävälleni Pekalle ja kaikille harrastuksieni kautta läheisiksi tulleille ystävilleni. Kaikkein eniten haluaisin kiittää poikiani Antonia, Patrikia ja Aleksia, joiden kautta pystyn näkemään oman elämäni arvot ja varsinkin vaimoani Rinaa, joka on vastaus kysymykseeni: “mikä on elämän tarkoitus?”.

LIST OF PUBLICATIONS

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2. Granström, T., Ojamo, H. and Leisola, M., Chemostat study of xylitol production by *Candida guilliermondii, Appl. Microbiol. Biotechnol*. **55** (2001) 36 - 42.

3. Granström, T. and Leisola, M., Controlled transient changes reveal differences in metabolite production in two *Candida* yeasts, *Appl. Microbiol. Biotechnol.* **58** (2002) 511 - 516.

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5. Granström, T., Aristidou, A. A. and Leisola, M., Metabolic flux analysis of *Candida tropicalis* growing on xylose in oxygen limited chemostat, *Metab. Eng.* (in press)

The author has been responsible for designing and carrying out all the experiments. Aristos Aristidou helped in interpreting the data for publications 1 and 5. Jouni Jokela helped in the analytical part of publication 1. Xiaoyan Wu helped in enzyme purification for publication 4 and Ulla Airaksinen carried out the microcultivation experiments. The author independently wrote the publications 1-3 and 5 and the other authors helped in refining the manuscripts. Matti Leisola who also was the supervisor for this thesis wrote the first version of publication 4.

ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CDW	Cell dry weight
CONLS	Constrained linear least square method
D	Dilution rate
DOT	Dissolved oxygen tension
k_{cat}	Reaction rate coefficient
K_I	Reaction inhibition coefficient
k_La	Oxygen transfer coefficient
K_m	Michaelis-Menten coefficient
LSF	Linear least square method
MFA	Metabolic flux analysis
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
PID	Proportional-Integration-Derivation process control
PO	The number indicating the efficiency of oxidative phosphorylation
PPP	Pentose phosphate pathway
PRPP	5-phosphoribosyl pyrophosphate
q(x)	specific production of biomass
Q(x)	volumetric production of biomass
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
XDH	Xylitol dehydrogenase
XK	Xylulose kinase
XR	Xylose reductase
YM	Yeast malt extract
YPD	Yeast peptone dextrose
Yxs	yield of biomass

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1 INTRODUCTION

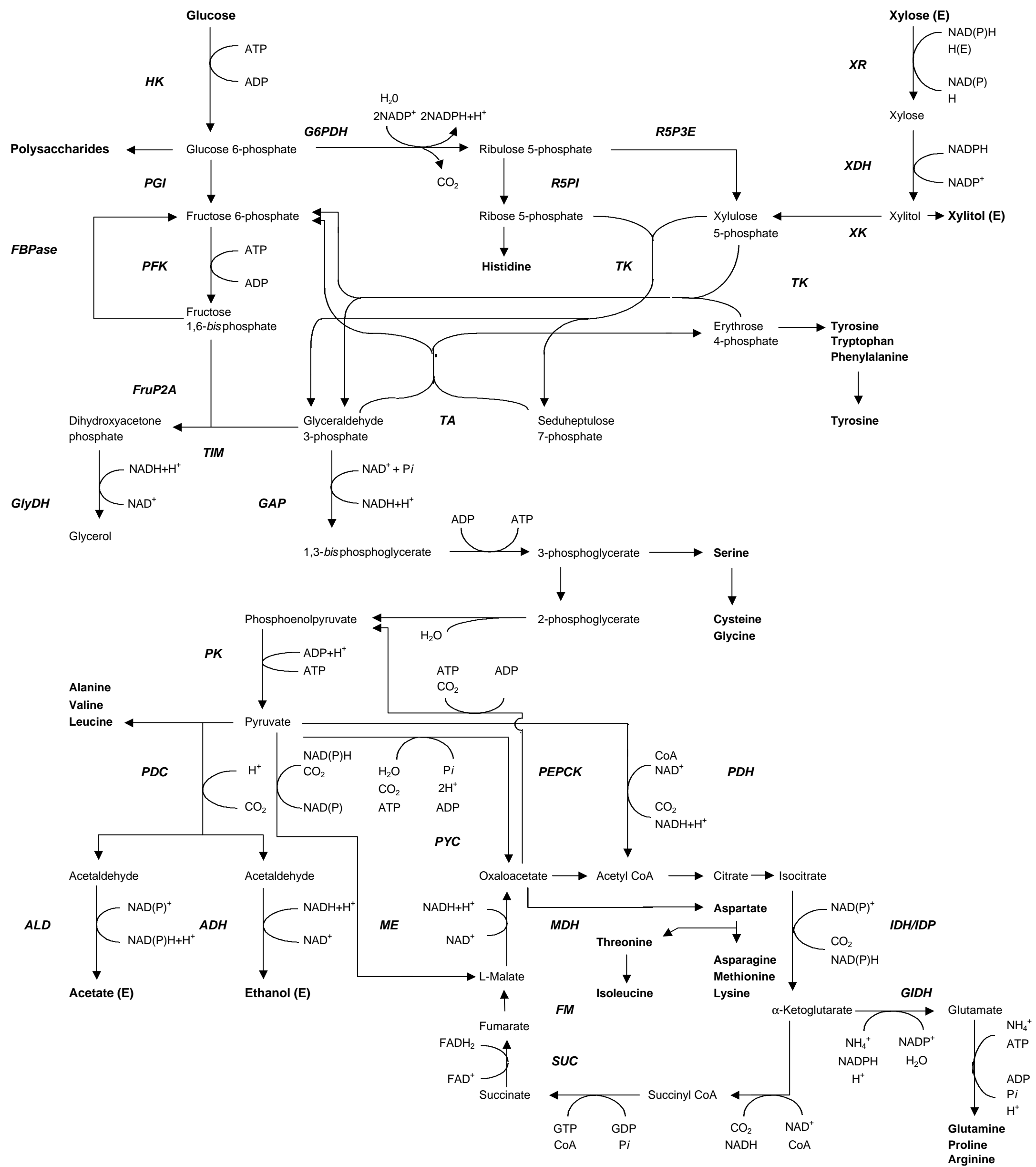
Originally, German and French scientists found xylitol around 1890. During the next five decades xylitol received very little attention. However, sugar shortages created by the Second World War forced to locate alternate sweeteners. Gradually, interest toward xylitol increased, when it's insulin-independent characteristics and involvement in essential pentosuria (harmless, genetic disorder) was revealed. Since 1970 xylitol has been studied intensively due to its benefits in preventing dental caries (Mäkinen, 2000; www.xylitolinfo.com).

Presently, xylitol is manufactured by reducing pure xylose, obtained from hard-wood hydrolysates, in the presence of a Raney nickel catalyst (Melaja *et al.* 1981). However, the chemical process requires several purification steps, because only pure xylose can be used for chemical reduction (Härkönen and Nuojua, 1979). Therefore, overall xylitol yield is relatively low (50 – 60 %) from the total xylan content of the wood hemicellulose (Nigam and Singh, 1995).

Xylitol production from xylose is a thoroughly studied subject in the literature. Excellent reviews, where readers may deepen their knowledge of the subject include microbial conversion of xylose to xylitol (Winkelhausen and Kuzmanova, 1998), xylose fermentation (Skoog and Hahn-Hägerdahl, 1987), utilisation of xylose (Jeffries, 1983), biochemistry and physiology of xylose fermentation (Hahn-Hägerdahl *et al.* 1994) and the history of xylitol (Mäkinen, 2000). We wanted to concentrate our studies to yeast cell physiology to find out the basic requirements for efficient xylitol production.

1.1 *Candida species* as model organisms for xylose metabolism

Candida spp. are an excellent set of model organisms for xylitol production study, because they have well-developed pentose phosphate pathway (PPP) and can grow on xylose as a sole carbon and energy source (Fig 1). They are organisms that are



Glucose
ATP
ADP
HK
Xylose (E)
NAD(P)H
H(E)
NAD(P)
H
XR
Xylose
XDH
NADPH
NADP+
G6PDH
R5P3E
Polysaccharides
Glucose 6-phosphate
Ribulose 5-phosphate
CO2
PGI
R5PI
Fructose 6-phosphate
Ribose 5-phosphate
Xylulose
5-phosphate
Xylitol
Xylitol (E)
XK
FBPase
PFK
Histidine
TK
Fructose
1,6-bisphosphate
Erythrose
4-phosphate
Tyrosine
Tryptophan
Phenylalanine
FruP2A
Dihydroxyacetone
phosphate
Glyceraldehyde
3-phosphate
TA
Seduheptulose
7-phosphate
TIM
NADH+H+
NAD+
GlyDH
Glycerol
GAP
NAD+ + Pi
1,3-bisphosphoglycerate
3-phosphoglycerate
Serine
Phosphoenolpyruvate
2-phosphoglycerate
H2O
Cysteine
Glycine
PK
ADP+H+
Alanine
Valine
Leucine
Pyruvate
PDC
H+
NAD(P)H
NAD(P)
PEPCK
CoA
PDH
Pi
2H+
PYC
Acetaldehyde
Oxaloacetate
Acetyl CoA
Citrate
Isocitrate
ALD
NAD(P)+
NAD(P)H+H+
ADH
ME
MDH
Aspartate
Threonine
IDH/IDP
Asparagine
Methionine
Lysine
L-Malate
Acetate (E)
Ethanol (E)
Isoleucine
GlDH
α-Ketoglutarate
Glutamate
FM
Fumarate
NH4+
NADP+
FADH2
SUC
FAD+
Succinate
Succinyl CoA
GTP
GDP
NADH
Glutamine
Proline
Arginine

Fig 1. Main metabolic pathways of glucose and xylose metabolism. HK = hexokinase, G6PDH = glucose 6-phosphate dehydrogenase, PGI = phosphoglucoisomerase, PFK = phosphofructokinase, FBPase = fructose 1,6 bisphosphatase, FruP2A = aldolase, TIM = triosephosphateisomerase, GlyDH = glycerol dehydrogenase, GAP = glyceraldehyde 3-phosphate, PK = pyruvate kinase, PDC = pyruvate decarboxylase, ALD = acetaldehyde dehydrogenase, ADH = alcohol dehydrogenase, PEPCK = phospho-*enol*-pyruvate carboxykinase, PYC = pyruvate carboxylase, PDH = pyruvate dehydrogenase, ME = malic enzyme, MDH malate dehydrogenase, IDH = isocitrate dehydrogenase (NAD), IDP = isocitrate dehydrogenase (NADP), GIDH = glutamate dehydrogenase, SUC = succinate dehydrogenase, FM = fumarase, XR = xylose reductase, XDH = xylitol dehydrogenase, XK = xylulokinase, TK = transketolase, TA = transaldolase, R5P3E = ribulose-5-phosphate-3-epimerase, R5PI = ribose-5-phosphate-isomerase.

someties placed among *fungi imperfecti* in the family *Moniliacae*. The PPP is divided into oxidative and non oxidative phase. The oxidative phase of PPP converts hexose phosphates to pentose phosphates providing NADPH required in biosynthetic pathways. The non-oxidative phase converts pentose phosphates into hexose phosphates and triglycerides. PPP provides also ribose-5-phosphate required for nucleic acid and histidine synthesis and erythrose-4-phosphate required for aromatic amino acid synthesis. The disadvantage attributed to the use of *Candida spp*. in food processing industry is due to the well-known human pathogen *Candida albicans*. However, genus *Candida* has an industrial and medical importance (Table 1). The Food and Drug Administration (FDA) in The United States has permitted *Candida guilliermondii* (ATCC 20474) and *Candida lipolytica* to be used in food for human consumption as **Secondary Direct Food Additives** in the production of citric acid.

1.2 D-Xylose metabolism in yeasts

In this section only the properties of *Candida spp*. are dealt with. However, if no references to *Candida spp.* exist in the literature also other species are discussed.

Table 1. Studied *Candida spp*. for food applications.

Process	Strain	Reference
Citric acid	*C. lipolytica*	Pazouki *et al.* 2000
Protein	*C. guilliermondii*	Halás and Lásztity 1991
L-arabitol from L-arabinose	*C. entomaea*	Saha and Bothast 1996
L-ascorbic acid from whey	*C. norvegensis*	Cayle *et al.* 1986
Lipase	*C. rugosa*	Dalmau *et al.* 2000
Vitamin C fatty acid esters	*C. antarctica*	Yan *et al.* 1999
Sophorolipid surfactant	*C. bombicola*	Deshpande and Daniels 1995

1.2.1 Uptake of xylose

Many *Candida* species uptake xylose by a specific carrier, with facilitated diffusion or by a proton dependent uptake (Jeffries, 1983; Hahn-Hägerdahl, 1994). The uptake of xylose has been studied *e.g.* with *Candida mogii* ATCC 18 364 (Sirisansaneeyakul *et al.* 1995). The xylose uptake rate followed Michaelis-Menten kinetics suggesting carrier mediated facilitated diffusion transport mechanism. This was confirmed with ^{14}C labelling studies. Alexander *et al.,* (1988) suggested that in *Candida shehatae* the aerobic xylose consumption is possibly transport limited, whereas in anaerobic conditions the NADH-linked xylose reductase is probably rate limiting. In non-starved cells D-xylose was transported with facilitated diffusion system with a K_m value of 125 mM in *C. shehatae* CBS 2779, however, starvation induced proton symport, in addition to facilitated diffusion system (Lucas and van Uden, 1986). *Candida utilis* possesses a low-affinity uptake system (K_m value of 67.6 mM) and a high affinity system (K_m value of 1.9 mM) for xylose transport, in addition to proton symport (Kilian *et al.* 1993).

1.2.2 Xylose metabolising pathway

The first three enzymes in xylose metabolising pathway in yeasts are D-xylose reductase (XR; E.C 1.1.1.21), xylitol dehydrogenase (XDH; E.C 1.1.1.9) and xylulokinase (XK; E.C 2.7.1.17) (Smiley and Bolen, 1982; Lachke and Jeffries, 1986; Slininger *et al.* 1987; Prior *et al.* 1989; Hahn-Hägerdahl *et al.* 1994). XR reduces xylose into xylitol and XDH oxidises xylitol into xylulose. In the last step before PPP XK phosphorylates xylulose into xylulose-5-phosphate. D-Xylose reductase can have a preference for both cofactors NADH and NADPH, but xylitol dehydrogenase is primarily NAD-dependent (Winkelhausen and Kuzmanova, 1998). NADP-dependent XDH activities have been reported in *C. utilis* and *P.stipitis* (Bruinenberg *et al.* 1984). Xylulokinase requires ATP (Fig 1). The thermodynamic equilibrum in XR and XDH catalysed reaction favours xylitol production (Fig 2). The K_{eq} (xylose $\Leftrightarrow$ xylitol) is 10^{10} and K_{eq} (xylitol $\Leftrightarrow$ xylulose) is $2.7 - 5.8^{-8}$ (Ditzelmuller *et al.,* 1984). Xylitol is produced in the oxygen limited conditions, where accumulation of NADH and subsequent inhibition of NAD-dependent XDH results in xylitol excretion (Smiley and Bolen, 1982; Bruinenberg *et al.,* 1984, Winkelhausen and Kuzmanova, 1998).

1.2.3 Xylose reductase

The enzyme has been isolated from *C. tropicalis* IFO 0618 (Yokoyama *et al.* 1995). Three isoforms of NADPH-dependent D-xylose reductases were purified and characterized. They had respective K_m values of 37, 30, and 34 mM for D-xylose and 14, 18, and 9 µM for NADPH, but NADH did not act as a cofactor. However, NADH dependent activities of XR have been measured from cell fee extracts (Horitsu *et al.,* 1992). The specificities of the three XRs for several aldoses were essentially the same. Both XR1 and XR2 were dimers composed of identical subunits and they had a molecular weight of 36 kDa. The pI values of XR1 and XR2 were 4.15 and 4.10, respectively. In addition XR has been purified from *C. shehatae* (Ho *et al.* 1990), *C. tenuis* (Neuhauser, 1997) and *C. intermedia* (Mayr *et al*, 2000). The gene coding for *C. guilliermondii* xylose reductase has been cloned and the protein expressed in *Pichia pastoris* (Handumrongkul *et al.* 1998). Similarly the xylose reductase of *C. tropicalis* IFO 0618 has been expressed in *Escherichia coli* (Suzuki *et al.* 1999). The

constructed strain was able to produce 13.3 g L^{-1} of xylitol during 20 h from xylose-glucose mixture.

1.2.4 Xylitol dehydrogenase

Yang *et al.* (1990) purified XDH from xylose-grown cells of *C. shehatae.* The purified enzyme had a molecular weight of 82 kDa and was composed of two subunits. In addition, the enzyme has been isolated from *P.stipitis* (Rizzi *et al.* 1989) and *Galactocandida mastotermitis* (Lunzer *et al.* 1998). Rizzi *et al*. (1989) concluded that NAD-dependent XDH is regulated by the catabolic reduction charge (NADH/[NADH + NAD^+]) and not by the total concentration of NAD^+ or NADH. When NAD^+ was the variable substrate and D-sorbitol concentration was held constant, NADH gave a competetive inhibition pattern at both non-saturating (1.2×10^{-5} M) and saturating (5×10^{-5} M) concentrations of D-sorbitol, respectively (Lunzer *et al.,* 1998). As an example, the intracellular concentration of NADH (0.39 µmol/g CDW) of *S. cerevisiae* growing in the glucose limited aerobic chemostat has been determined by Lange *et al.* (2001). Girio *et al.* (1996) purified and characterised the XDH from *Debaryomyces hansenii.* The enzyme was most active at pH 9.0-9.5 and the K_m values for xylitol and NAD were 16.5 and 0.55 mM, respectively. Ca^{2+}, Mg^{2+}, and Mn^{2+} did not affect enzyme activity, conversely, Zn^{2+}, Cd^{2+}, and Co^{2+} strongly inhibited the enzyme.

1.2.5 Xylulokinase

D-Xylulokinase has been purified from *Pichia stipitis* NCYC 1541 (Flanagan and Waites, 1992). The molecular mass of the native enzyme is 120-130 kDa and it contains two identical subunits of 71 kDa. The enzyme requires magnesium ions for optimal activity and it was found to lose all its activity after 24 h at 4 °C. Yang *et al* (1997) studied the induction of xylulokinase activity of *C. shehatae* and *Pachysolen tannophilus* by growing cells on glucose and xylose. The patterns of induction and inactivation of hexokinase, phosphofructokinase, and xylulokinase enzyme titers indicated that the basic regulatory mechanisms differ in the two xylose-fermenting yeasts. Deng and Ho (1990) isolated, cloned and overexpressed the gene encoding

xylulokinase in *S. cerevisiae* resulting two-fold increase in xylulose utilisation. Rodriguez-Peña *et al.* (1998) deleted the xylulokinase encoding sequence in the genome of *S. cerevisiae.* The resulting strain could not grow on D-xylulose as the sole carbon source. The overexpression of xylulokinase resulted in a strain, which had normal growth on glucose, whereas impaired growth on D-xylulose. It can be speculated that overexpression of an ATP consuming enzyme (in this case XK) will result in ATP depletion due to increased uptake of substrate. This has been suggested also by Teusink *et al.* (1998). They showed by computer simulation that overexpression of hexokinase enzyme will result in ATP depletion. Conversely, overexpression of XK gene in *S.cerevisiae*, which also expresses XR and XDH from *P.stipitis* resulted in enhanced the specific xylose utilisation (Toivari *et al.,* 2001). This suggests that the order of enzymes in the metabolic pathways and their cofactor or energetic dependencies has to be taken into consideration, when overexpressing enzymes.

1.3 Xylose reduction to xylitol

Reduction of xylose to xylitol has been 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; Vandeska *et al.,* 1996). In addition, to the known mechanism of XDH inhibition by intracellular NADH, various process variables like size of inoculum, substrate concentration, medium composition, temperature, pH, aeration and other environmental factor have an impact on xylitol production. However, only oxygen availability, substrate concentration and cosubstrate feeding are reviewed here, because, they are consistent with the topic of this research.

1.3.1 Oxygen availability

One of the most critical parameters in xylitol accumulation is oxygen availability. Under oxygen-limited conditions the difference in the cofactor requirements of the XR and XDH causes a redox imbalance, which influences xylitol accumulation in yeasts (Bruinenberg *et al.* 1983). Xylitol formation is favoured under oxygen-limited

conditions, because of the NADH accumulation and subsequent inhibition of NAD-linked XDH (van Dijken and Scheffers, 1986). However, a small amount of oxygen is required for ATP and cofactor production, otherwise XK may become a bottleneck in xylose consumption due to ATP depletion. It has hitherto not been shown that xylose could be a sole carbon source in anaerobic conditions either in *Candida* yeasts or in a recombinant *S. cerevisiae* on a strictly mineral medium. Theoretically, it can be predicted that there is an optimal oxygen consumption rate for each individual strain in respect to maximal xylitol production. Most of the studies published represent this approach (see review by Winkelhausen and Kuzmanova, 1998). Several studies have been made on the effect of oxygen on xylitol production. Vandeska *et al.* (1995) obtained maximum xylitol production with *C. boidinii* at OTR of 14 mmol L^{-1} h^{-1}. Oh *et al.* (1998) maximised the xylitol production of *C. parapsilosis* at a redox potential of 100 mV. Girio *et al.* (1994) studied the effect of different OTR on xylitol and ethanol production of *D. hansenii*. The highest production rates were achieved with the OTR value of 0.045 mmol L^{-1} h^{-1}.

1.3.2 Substrate concentration

In addition to aeration, the activities of XR and XDH are also sensitive to substrate concentration. Since the real substrates in industrial xylitol production are lignocellulosic hydrolysates, which often contain a variety of sugars (D-xylose, D-glucose, D-mannose, D-galactose, L-arabinose etc), it is important to investigate their influence on the induction of XR and XDH enzyme activities. Sugai and Delgenes (1995) found that D-Xylose and L-arabinose were the best inducers of both XR (NADPH dependent) and XDH in the cell free extracts of *C. guillermondii* NRC 5578, but only negligible activities were observed if D-glucose served as carbon source. When D-xylose and L-arabinose were present in a mixture, the consumption of both pentoses was reduced by the presence of the second sugar, although both sugars were consumed simultaneously by cells. They concluded that the expression of XR is under a catabolite repression control (Sugai and Delgenes, 1995). Kern *et al.* (1997) studied the ability of various sugars and sugar alcohols to induce xylose reductase and xylitol dehydrogenase activities in the yeast *C. tenuis* CBS 4435. Both enzyme activities were induced, when the organism was grown on D-xylose, L-

arabinose, D-arabinose and D-lyxose. A mixture of D-xylose with the more rapidly metabolizable sugar D-glucose resulted in a decrease in the levels of both enzymes formed. These results show that the utilization of D-xylose by *C. tenuis* is regulated both induction and catabolite repression.

1.4 Different strategies for xylitol production

Eventhough *Candida spp*. are the most efficient xylitol producers their use in food industry is contradictory due to the pathogenic nature of *C. albicans*. Therefore, xylitol producing recombinant *Saccharomyces cerevisiae* have been studied intensively to replace *Candida spp*. as a production organisms. However, use of recombinant organism may pose a similar problem for food industry than using a potential opportunistic pathogen microbes. In order to avoid all the imago problems xylitol can be produced with purified enzymes, also.

1.4.1 Metabolically engineered *Saccharomyces cerevisiae*

An efficient conversion of xylose to xylitol was achieved by Hallborn *et al*. (1991). They transformed *Saccharomyces cerevisiae* with the gene encoding the xylose reductase (XR) of *Pichia stipitis* CBS 6054. A fed batch cultivation was carried out with glucose as the growth substrate and glucose-xylose (19:1 g L^{-1}) solution as a feed. *S. cerevisiae* transformed with the XYL1 gene gave over 95 % conversion of xylose into xylitol (Hallborn *et al.,* 1991). Using the same strain the influence of a cosubstrate and aeration on xylitol production was studied by Hallborn *et al.* (1994). With either glucose or ethanol as a cosubstrate the conversion yields were close to 1 g xylitol g $xylose^{-1}$, however the volumetric productivities were below 0.23 g L^{-1} h^{-1}. Oxygen limitation increased the xylitol yield, but decreased the productivity.

Also Meinander *et al.,* (1994) used the same recombinant *S. cerevisiae* strain as Hallborn *et al.,* (1991 and 1994). They studied the bioconversion of xylose into xylitol in fed-batch fermentation. When only xylose was fed into the fermentor, the

production of xylitol continued until the ethanol that had been produced during an initial growth phase on glucose, was depleted. It was concluded that ethanol acted as a redox balance maintaining cosubstrate. The conversion of high amounts of xylose into xylitol required ethanol to the feed solution. Under oxygen limited conditions, acetic acid accumulated in the fermentation broth. The best yield (2.4 g xylitol g ethanol^{-1}) was achieved under oxygen-limited conditions (Meinander *et al.* 1994).

1.4.2 Xylitol production *in vitro*

Neuhauser *et al.* (1998) devised an *in vitro* process with pure and soluble enzymes. Formate dehydrogenase (FDH) was supplied with formate thus enabling an NADH dependent reduction of xylose to xylitol by XR continuously. The enzymatic synthesis of xylitol was carried out at pH 7 at 25 °C in 20 mL bioreactor. Fed-batch conversion of 0.5 M (76 g L^{-1}) xylose into xylitol yielded productivity of 2.8 g L^{-1} h^{-1} during 20 h.

1.5 Xylitol production characteristics of the studied *Candida species*

The xylitol production characteristics of three different *Candida* strains were studied in this work. *C. milleri* was the only strain that was already used in food processing industry, but its physiology and characteristics was almost unknown. The xylitol production characteristics of *C. guilliermondii* and *C. tropicalis* used in this study was already screened by Ojamo (1994) in the shake flask. However, no thorough physiological or quantitative studies had been carried out.

1.5.1 Candida milleri

C. milleri has been used in sourdough rye baking in Finland as part of a mixed culture together with lactic acid bacteria for centuries. It is an acid tolerant food grade organism and thus ideal for production of xylitol type sweeteners. According to Mäntynen *et al.* (1999) *C. milleri* is taxonomically closely related to *Saccharomyces cerevisiae*, which could facilitate its genetic modification. *C. milleri* has not been used

in xylitol production studies before. Due to its background in sourdough baking it was decided to be included in this research.

1.5.2 *Candida guilliermondii*

C. guilliermondii was chosen on the basis of its high xylitol production capacity. Ojamo (1994) demonstrated that *C. guilliermondii* (VTT-C-71006) is an efficient xylitol producer. A xylitol yield of 0.74 g g^{-1} xylose was obtained within 50 h at an initial xylose concentration of 100 g L^{-1}. Using a fed batch cultivation the xylitol yield was increased to 0.78 g g^{-1} and process time was decreased by 40 %, in addition the initial xylose concentration could be increased to 250 g L^{-1} (Ojamo, 1994). Roberto *et al.* (1999) studied the effect of k_La on the conversion of xylose to xylitol by *C. guilliermondii* (FTI 20073) in batch fermentation. With rice straw hydrolysate as a substrate (containing 62 g L^{-1} of xylose) the maximum volumetric productivity was 0.52 g L^{-1} h^{-1} and the highest xylitol concentration (36.8 g L^{-1}) was attained at k_La 15 h^{-1} 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 0.91 and 0.58 g L^{-1} h^{-1} respectively. Barbosa *et al.* (1988) achieved 77.2 g L^{-1} xylitol concentration from 104 g L^{-1} of xylose with *C. guilliermondii* FTI-20037 using shake flasks with high cell density and defined medium. Meyrial *et al.* (1991) achieved 221 g L^{-1} xylitol yield from 300 g L^{-1} of xylose with average specific production rate of 0.19 g g CDW^{-1} h^{-1} in the shake flask study.

1.5.3 *Candida tropicalis*

C. tropicalis is often considered to be an opportunistic pathogen, which limits its use in food processing industry. It has potential industrial importance, because of its high xylose uptake rate, xylitol production capacity and alkane and fatty acid degradation in its peroxisomes. Its morphology changes from mycelium culture to single cell culture according to oxygen availability in the chemostat (data not shown). *C. tropicalis* is one of the most common model organisms in xylitol production studies.

Table 2. Summary of xylitol production studies with *Candida species*.

Strain	Yield $g\ g^{-1}$	Initial xylose $g\ L^{-1}$	Productivity $g\ L^{-1}\ h^{-1}$	Process strategy	Ref.
C.guilliermondii	0.78	250	nr	Fed batch oxygen limit.	Ojamo, 1994
C.guilliermondii	0.73	62	0.52	Batch, oxygen limit.	Roberto *et al.,* 1999
C.guilliermondii	0.2	15.5	0.58	Packed bed reactor	Domínguez *et al.,* 1999
C.guilliermondii	0.74	104	nr	Shake flask 100 ml	Barbosa *et al.,* 1988
C.guilliermondii	0.75	300	nr	Shake flask 500 ml	Meyrial *et al.* 1991
C.tropicalis	0.82	750	4.94	Cell recycling Yeast exctract Glu-xyl feed O_2 limit	Choi *et al.,* 2000
C.tropicalis	0.64	172	2.67	Yeast extract, O_2 limit.	Horitsu *et al.,* 1992
C.tropicalis	0.91	300	3.98	Glu-xyl feed O_2 limit.	Oh and Kim, 1998
C.tropicalis	0.82	127	3.26	fed batch glu-xyl feed O_2 limit.	Yahashi *et al.,* 1996
Saccharomyces cerevisiae (recombinant)	0.95	190	0.40 (calc)	fed batch glu-xyl feed	Hallborn *et al.,* 1991

nr = not reported; (calc) = calculated from the values given in the article

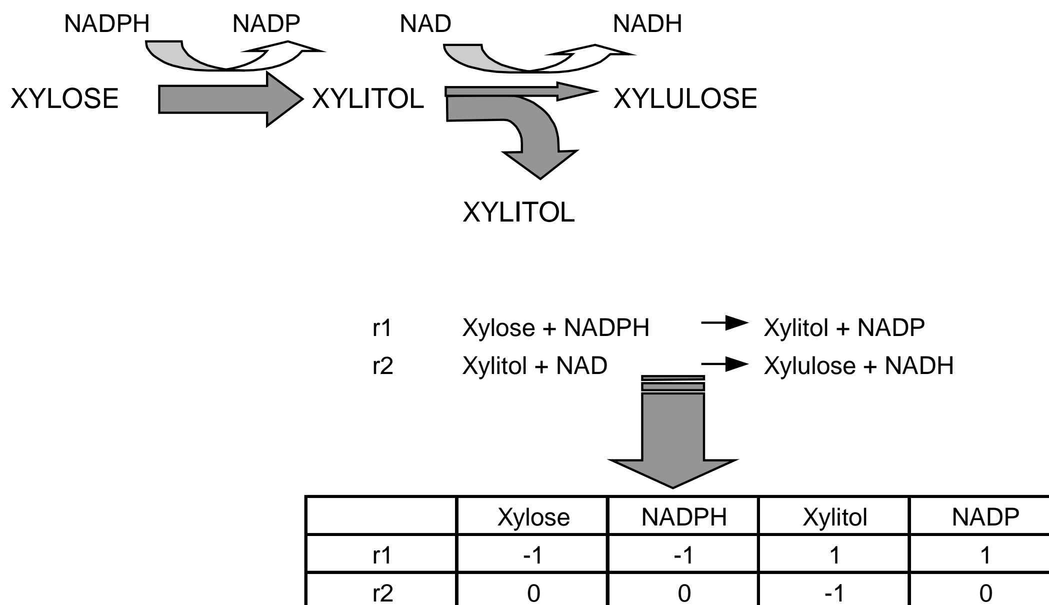
Production of xylitol in cell recycle fermentations by *C. tropicalis* ATCC 13803 was studied by Choi *et al.* (2000). The cell-recycling studies indicated that the feeding of xylose, glucose and yeast extract in the xylitol production phase was most effective in enhancing xylitol productivity. It resulted in 0.82 g xylitol g $xylose^{-1}$ yield and 4.94 g xylitol L^{-1} h^{-1} productivity. Horitsu *et al.* (1992) studied the effect of culture conditions on xylitol production rate with *C. tropicalis*. They achieved a maximum xylitol production rate of 2.67 g $L^{-1}h^{-1}$, when initial xylose and yeast extract concentration were 172 and 21 g L^{-1}. Oh and Kim (1998) carried out feeding experiments with different ratios of xylose and glucose and their effect on xylitol production of *C. tropicalis*. The maximum xylitol yield from 300 g L^{-1} of xylose was 91 % at a glucose / xylose feeding ratio of 15 %, while the maximum volumetric production rate of xylitol was 3.98 g L^{-1} h^{-1} at a glucose / xylose feeding ratio of 20 %. Yahashi *et al.* (1996) studied the xylitol production with *C. tropicalis* using a fed batch cultivation and glucose as a cosubstrate. Xylitol was produced with a yield of 0.82 g g^{-1} xylose consumed and with volumetric production rate of 3.26 g L^{-1} h^{-1}. Faster growth occurred when a mixture of glucose and xylose was used as a substrate. Azuma *et al.* (2000) studied the effect of salt on xylitol production by *C. tropicalis*. An addition of 4 % NaCl increased the yield of xylitol from 5 % D-xylose by 1.3-fold. The addition of salts also increased the xylitol production even in the presence of 2 % D-glucose. They suggested that the increase in xylitol production by NaCl may be due in part to the increase in xylose reductase production. Production of xylitol with *C. tropicalis* has also recently been patented in Japan (JP 2000093188 and JP 3007615).

A summary of the results are presented in the table 2. It can be concluded that different growth conditions employed in the studies makes comparison between different strains and species difficult. Volumetric productivity (g L^{-1} h^{-1}) is used, eventhough specific productivity (g CDW^{-1} h^{-1}) would give a better comparison. This is due to the fact that in many articles, biomass concentration is not given.

1.6 Modelling of metabolism

1.6.1 Constructing the metabolic network

Only a short review of metabolic flux analysis is given here (Table 3). A more thorough treatment of the topic can be found in standard textbooks (Vallino and Stephanopoulos, 1990; Stephanopoulus *et al.* 1998). The first step in the development of a metabolic network is to extract the relevant reactions that represent the major carbon fluxes, ATP turnover and NAD(P)H consuming or generating reactions (Fig 2).



	Xylose	NADPH	Xylitol	NADP
r1	-1	-1	1	1
r2	0	0	-1	0

Fig 2. Constructing the metabolic network matrix. The reactions included in the matrix are placed in the rows of the matrix. Each metabolite is given an individual number and they are placed in the columns of the metabolic matrix. In case of double dependent enzyme, both cofactors (NADH/NADPH) can be included in the metabolic matrix, but with different stoichiometric coefficient. This can be determined, for example *in vitro* enzyme assays.

If there are two linearly dependent reactions in the metabolic matrix (singularity problem) these reactions are lumped together. Very often this is the case with biomass

Table 3. Flow sheet for constructing the metabolic stoichiometric matrix.

System Summary: $\underline{\mathbf{A}}\ \underline{\mathbf{x}} = \underline{\mathbf{r}}$
Metabolic stoichiometric matrix, $\underline{\mathbf{A}}$ $(n \cdot m)$
n metabolites, m reactions (stoichiometric coefficients)
Rate Vector $\underline{\mathbf{r}}$ $(n \cdot 1)$; $r^T = [r_1,\ldots\ldots r_n]$; (C-mol / L / h)
measured (biomass, ethanol, CO_2, etc.)
intracellular metabolites in pseudo-steady-state, $r_i = 0$
Metabolic flux vector $\underline{\mathbf{x}}$ $(m \cdot 1)$
$\mathbf{x}^T = [x_1,\ldots\ldots x_m]$; (C-mol / L / h)
Dependency test:
rank (A) = m (linearly independent rows = number or reactions
rank (A) < m
linearly dependent reactions => lumped together
Three scenarios:
m = n, A square full rank => $x = A^{-1} r$ (simple matrix inversion)
m > n, over-determined system (equations > unknowns)
=> $x = (AA^T)^{-1} A r$ (least square fit solution)
m < n, under-determined system (unknowns > equations)
linear programming: minimize or maximize particular objective function, e.g. max growth rate numerical tool box available, e.g LINPAK

synthesis and maintenance requirements. Once the metabolic matrix (**A**) is constructed the production rate vector **r** must be determined. The **r** vector contains all the measured metabolites and all the rates of intracellular metabolites. The intracellular metabolites are set to zero *ie*. no accumulation will occur (PSS = pseudo steady state). The matrix equation for the rate of change of each metabolite in the metabolic network is presented:

$$\mathbf{A}\ \mathbf{x} = \mathbf{r} \qquad (1 - 1)$$

where **A** is the metabolic matrix constructed with all the relevant biochemistry based on stoichiometry, **r** is the production rate vector for all the metabolites in the network and **x** is the unknown flux vector to be determined (Table 3).

1.6.2 Metabolic flux analysis (MFA)

For studying intracellular fluxes a number of different analytical methods can be used. These include mass spectrometry with MFA (Wittman and Heinzle, 1999), NMR spectroscopy (Petersen *et al.,* 2000; Frey *et al.,* 2001; Schmidt *et al.,* 1999; Graaf *et al.,* 1999) and MFA and/or *in vitro* enzyme assays (Vallino and Stephanopoulos, 1993 and 1990; Nissen *et al.,* 1997; Gulik and Heijnen, 1995; Jong-Gubbels *et al.,* 1996; Hoek *et al.* 1998). MFA can be used for identification of branch points of pathways, calculation of nonmeasured extracellular fluxes or calculation of maximum theoretical yields (Stephanopoulos *et al.* 1998). One disadvantage of MFA is that intracellular substrate cycling and equilibrum reactions are difficult to model without running into singularity problems in matrix calculations. To certain extent this problem can be overcome in eukariotic cells by placing the same metabolite in different cell compartments. If only the main pathways are included in the metabolic network the results may be self-explanatory. Furthermore, it is impossible to extend the applicability of stoichiometric modelling in calculating the cofactor balances for e.g. ATP, NADH or NADPH. MFA facilitates analysis and more importantly comparison of large sets of data in different conditions.

1.6.3 Examples of using MFA in modelling yeast metabolism

Nissen *et al.* (1998) have studied flux distribution in anaerobic, glucose limited chemostat. They were able to show a redox shunt across the inner mitochondrial membrane consisting of the reactions catalysed by mitochondrial and cytosolic ADH. The metabolism of a vitamin-auxotrophic pyruvate-producing microorganism,

Torulopsis glabrata was investigated by Hua *et al*. (1999). MFA indicated that the thiamine concentration significantly affected pyruvate dehydrogenase and pyruvate decarboxylase activities. MFA was also utilized to clarify the metabolism of this strain during pyruvate fermentation under different oxygen supply conditions. Based on the analysis of thiamine concentration on the metabolic fluxes, the authors conducted a fed-batch experiment where the overall pyruvate yield could be improved by 15% due to the decrease of ethanol production (Hua *et al.,* 1999). Urrieta-Saltijeral *et al.* (2001) studied flux distribution for a wild and a mutant strain of *Saccharomyces cerevisiae* in anaerobic conditions. Comparative analysis of carbon split in the metabolic network for the mutant yeast strain lacking the G6PDH and for the reference strain allowed to conclude that the PPP is in priority devoted to its anabolic function rather than to the production of NADPH cofactors. This last function seems to be as well assumed by the specific NADP acetaldehyde dehydrogenase enzyme. According to them this explains the significantly higher production of acetate by the mutant strain (Urrieta-Saltijeral *et al*, 2001). Granstöm *et al.* (2000) carried out MFA, which took into consideration yeast cell compartmentation and cofactor dependencies of *in vitro* measured enzyme activities of *C. millerii*. It was also possible to calculate the differencies in ATP yield, when *C. millerii* was growing on a different carbon sources. This was done by including all the main reactions involving ATP, NADH or NADPH in the metabolic matrix. Additional constraints were imposed on the flux estimates such that the directionality of irreversible reactions is not violated.

2 AIMS OF THE STUDY

One advantage of using a microbiological xylitol production is the possibility to use industrial sidestreams as a raw material, which contains hemicellulose fractions without any multiple purification steps. This enables better use of renewable natural resources in the future. The aim of this work was to develop a biotechnological process for xylitol production. In addition, to study the xylose catabolism in *Candida* yeasts with specific implication to xylitol production. Three potential *Candida* yeasts have been compared in their ability to produce xylitol from xylose. This work can be divided into three different main goals:

1. to find out the most productive cultivation mode in xylitol production
2. to study the biochemistry of xylose metabolism in *Candida* yeasts
3. to develop a tool to model xylose metabolism through computer simulation

Is xylitol production dependent on biomass growth in *Candida spp*? What is the implication of cosubstrate for xylitol production? What is the most optimum oxygen level for xylitol production? These questions needed to be solved to determine the most productive cultivation mode *ie.* batch, fed batch or chemostat? Using this information it is possible to find out the maximum flux-split ratio between PPP and xylitol flux. In order to reveal the metabolic bottlenecks in xylitol production all bioreactor cultivations were decided to carry out on mineral medium, no additional growth supplements *ie*. yeast extracts added. The question of source of cofactors (NADPH/NADH) during xylitol production was studied by *in vitro* enzyme assays. In addition, we were interested to find out, whether xylitol production from xylose served as a redox sink as response to oxygen limited conditions - similarly to ethanol production from glucose in anaerobic conditions. Finally, we wanted to develop a metabolic flux model, which could take into consideration the yeast cell physiology *ie*. compartmentation and cofactor dependency. This model would facilitate the comparison between different growth conditions and carbon sources in respect to xylitol production.

3 MATERIALS AND METHODS

Only a general outline of the methods are presented here. A more detailed description of the used materials and methods can be found in the respective publications (I – V) .

3.1 Organism, maintenance and inoculum preparation

Candida tropicalis VTT-C-78086 (ATCC 1369), *Candida guilliermondii* VTT-C-71006 and *Candida milleri* VTT-C-95232 were obtained from VTT Biotechnology (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 YPD-medium at 30 °C and 200 rpm. YPD-medium contained 10 g/L yeast extract (Difco), 20 g/L bactopeptone (Difco) and 20 g/L glucose (Fluka).

3.2 Chemostat experiments

Mineral medium was prepared according to Verduyn *et al.* (1992). The medium 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 mg, $ZnSO_4 \cdot 7H_2O$ 4.5 mg, $CoCl_2 \cdot 6H_2O$ 0.3 mg, $MnCl_2 \cdot 4H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 0.3 mg, $CaCl_2 \cdot 2H_2O$ 4.5 mg, $FeSO_4 \cdot 7H_2O$ 3 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.4 mg, H_3BO_4 1 mg, KI 0.1 mg and silicon based antifoam agent 0.05 mg. When necessary all the medium components were doubled due to high concentration of xylose in the medium. Mineral medium was autoclaved for 20 min at 120°C. After autoclaving a filter sterilised vitamin solution was added giving a final concentration per litre of biotin 0.05 mg, calcium panthotenate 1 mg, nicotinic acid 1 mg, *myo*-inositol 25 mg, pyridoxal hydrochloride 1 mg and *para*-aminobenzoic acid 0.2 mg. Glucose and xylose were sterilised at 110 °C for 20 min and added separately to the growth medium in order to give a final concentration of either 7.5 g L^{-1} (0.25 C-mol L^{-1}) or 50 g L^{-1} (1.67 C-mol L^{-1}) depending on the experiment. Culture purity was monitored on a regular basis by phase contrast microscopy. The formic acid solution was

prepared by adding formate after autoclavation to a final concentration of 115 g L^{-1} (2.5 C-mol L^{-1}).

Chemostat cultivations were carried out in a 2-litre fermenter (B.Braun MD) on the above mentioned mineral medium at 30 °C and the culture pH was set at 5.0 and it was kept constant by addition of 2M KOH. Fermenter was installed on a balance and it was calibrated with 1 L medium inside. The working volume of 1000 ml was kept constant by removing effluent from the bottom of the fermenter with a peristaltic pump (Watson-Marlow 505U) that was connected to a PID-controlled balance. Substrate solution was fed into fermenter with peristaltic pump (Watson-Marlow 101U). The actual working volume was determined at the end of each experiment. The airflow rate was set to 0.888 L/min and it was controlled using a massflow controller (Bronkhurst HiTec, Ruurlo, Holland). The dissolved oxygen tension (DOT) was measured with O_2-electrode (Ingold). In all aerobic cultures the DOT was over 30 % to ensure fully aerobic conditions. Transient conditions were created by changing the agitation and dilution rates simultaneously (articles III and V). Generally, five volume changes were allowed to take place before analyses and all the growth parameters were measured on two successive days to ensure steady state conditions. Cofeeding of formate was done with a peristaltic pump (Watson-Marlow 101U), which was controlled by a DCU unit of the B.Braun MD fermenter.

3.3 Exhaust gas analysis

The fermentation exhaust gas was cooled to 4 °C in a condenser to prevent the evaporation of volatile compounds before entering into the masspectrometer (VG-Prima 600). Carbon dioxide, oxygen, argon and nitrogen were analysed from exhaust gas. In calculating the oxygen consumption rates and carbon dioxide production rates temperature of 30 °C was assumed and air pressure was taken from the daily weather forecast.

$$\Delta n_g = k \cdot p \cdot \Delta V_g / R \cdot T \qquad (1\text{-}2)$$ where

k = constant for balancing the inlet and outlet gas streams V_g^{in} / V_g^{out} (argon)

Δn_g = consumption of oxygen [mmol/h]

p = pressure [Pa] = 101325 Pa

ΔV_g = consumption of oxygen [m^3/h] = $V_{in, O2} - V_{out, O2}$

R = 8.314 J/mol·K

T = temperature [K] = 293.15 K

3.4 Cell dry weight measurements

Culture samples (5 ml) were vacuum filtered through preweighed nitro-cellulose filters (0.45 µm, Schleicher & Schuell), washed with Milli-Q water and then dried in a microwave oven for 20 min (Ignis, Japan) and weighed. All the assays were done in duplicate.

3.5 Substrate and metabolite analysis

Samples (1.2 ml) from chemostat were centrifuged at 8000 **g** for 5 min (Heraeus Sepatech, Biofuge A, Germany) and the supernatant was analysed. Glucose, xylose, formate, xylitol, glycerol, acetate and ethanol concentrations were analysed by 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. All the metabolites were visible with RI detector except formate, which was analysed by UV (214 nm). Serie of standard solution mixture containing all other metabolites except formate was used (g L^{-1}): 0.05; 0.1; 0.25; 0.5; 1.0; 2.0; 5.0. Same kind of serie was done to formate separately (Articles I, II, III, IV and V).

3.6 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 Vibra Cell sonicator (VCX600, USA) equipped with microtip probe (13 mm). Ten 30 s pulses were applied with 30 s

cooling period between each pulse. All the reagents and equipments were kept in ice during sonication.

3.7 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. One unit of activity is defined as the amount of enzyme catalysing the conversion of one µmol substrate per minute. Specific activities are expressed as units (U) per mg protein.

3.8 Protein determination and ammonia assay

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

Ammonium was measured enzymatically by means of glutamate dehydrogenase (GlDH; EC 1.4.1.3) with NADPH as a cofactor according to Bergmeyer (1985). The assays were performed with a Shimadzu UV-2100 spectrophotometer.

3.9 Purification of xylose reductase

Xylose reductase from the cell-free extract of *C. guilliermondii* was purified by a BioPilot System using DEAE Sepharose Fast Flow, Phenyl Sepharose 6 Fast Flow (high substitution) and Sephadex G-75 columns (Pharmacia). All the purification steps were carried out at 4-8 °C. The cell-free extract from *C. guilliermondii* was loaded onto a DEAE-Sepharose Fast Flow column equilibrated with potassium phosphate buffer (100 mM, 1 mM 1,4-dithiothreitol, 2 mM $MgCl_2$, pH 7.5; buffer A). Xylose reductase was eluted with an increasing NaCl gradient at 2.5 ml/min. Fractions containing xylose reductase activity were pooled and concentrated by

ultrafiltration and then applied to a Sephadex G-75 column equilibrated with buffer A and eluted at a flow rate of 0.5 ml/min. The xylose reductase active fractions obtained from gel filtration were combined and $(NH_4)_2SO_4$ was added to give a concentration of 1 M. The enzyme solution was then applied to a Phenyl Sepharose Fast Flow column previously equilibrated with 50 mM potassium phosphate buffer containing 1 mM 1,4-dithiothreitol and 1 M $(NH_4)_2SO_4$ (pH 7.5). Xylose reductase was eluted by a decreasing $(NH_4)_2SO_4$ gradient.

3.10 Constructing the metabolic flux analysis

Localisation and cofactor specificity of assayed enzymes were taken into account in constructing the metabolic network. The matrices are full rank *ie.* the biochemical reactions are not linearly dependent on each other. Since this is an overdetermined system, the solution can be found by a least-square-fit (LSF) method.

In order to increase the applicability of the model it was extended to include all the main reactions involving ATP, NADH or NADPH. Contrary to the other published MFAs the measured oxygen consumption and carbon dioxide production rates were included into the model, in addtion to other measured parameters. The NADH / NADPH ratio of individual enzymes was determined by *in vitro* enzyme assays. Additional flux coefficients for substrate uptake were imposed into the model, consequently constrained linear least-squares (CONLS) method has been used. Solving the problem according to LSF method, **x** is found according to the following equation:

$$\mathbf{A}\,\mathbf{x} = \mathbf{r} \quad \Rightarrow \quad \mathbf{x} = (\mathbf{A}^{\mathbf{T}}\mathbf{A})^{-1}\,\mathbf{r} \qquad (1 - 3)$$

where **A** is the stoichiomeric matrix of biochemical reactions. Vector **r** contains the net accumulation rates and vector **x** contains the fluxes. The solution for constrained problem is subjected to following condition:

$$\mathbf{A\,x = r} \;\; => \;\; \textbf{where} \quad \mathbf{C\,x < d} \qquad (1 - 4)$$

The matrix **C** determines the reactions whose flux must equal or be less than **d**. In other words matrix **C** determines the direction of the constrained reactions and matrix **d** determines the numerical value of the constraint.

$$\mathbf{Ax = r + (A^T)^{-1} C^T d}$$

both sides are multiplied with $\mathbf{A^T}$**:**

$$\mathbf{A^TAx = A^T r + A^T(A^T)^{-1} d} \quad \Leftrightarrow \quad \mathbf{A^TAx = A^T r + I\,d}$$

where **I** is identity matrix. In order to solve **x**, both sides are multiplied with $\mathbf{(A^TA)^{-1}}$**:**

$$\mathbf{(A^TA)^{-1}(A^TA)\;x = (A^TA)^{-1}A^T\;r + (A^TA)^{-1}\;d}$$

$$\mathbf{x = (A^TA)^{-1} A^T r + (A^TA)^{-1} C^T d} \qquad (1 - 5)$$

The solution to the problem refer to as inequality constrained least-squares according to Vallino and Stephanopoulos (1990) and it must be solved numerically. However, we used commercial software of constrained linear least-squares (CONLS) method by MATLAB (The MathWorks Inc). The metabolic network was constructed by Mathcad 2000 Professional (MathSoft Inc).

4 RESULTS

4.1 Xylitol production by *C. milleri* (I)

Xylitol production characteristics of *C. milleri* was studied in the shake flask and in the chemostat with glucose or glucose-xylose mixture as a carbon source. Both fully aerobic and oxygen limited conditions were studied in the chemostat on a mineral medium, but complex medium was used in the shake flask experiments. *In vitro* enzyme assays were carried out from the glucose and glucose-xylose cultivations. MFA was done based on the measured data from the glucose and glucose-xylose cultivation.

4.1.1 Shake flask and chemostat experiments

C. milleri did not effectively convert xylose to xylitol even in the presence of glucose although low concentrations of xylitol were detected. In shake flasks some xylitol (0.2 g L^{-1}) accumulated in the presence of glucose-xylose mixture and xylose alone. Furthermore, some growth on xylose could be detected on YM-medium (data not shown). The shake flask experiments indicated that *C. milleri* was not able to consume xylose as a sole carbon source. Therefore, a series of chemostat runs were carried out with varying ratios of glucose and xylose as a carbon substrate starting from 10 % glucose and 90 % of xylose and consequent increase in glucose and reduction in xylose concentration. It was assumed that glucose, as a cosubstrate would facilitate the consumption of xylose and regenerate cofactors and energy for xylitol production.

In these steady states no detectable xylose consumption or xylitol production was measured. However, a small amount of xylitol (7.2 C-mmol /L) was produced with the glucose to xylose ratio of 1:1 in fully aerobic conditions (Granström, *et al.,* 2000). *C. milleri* was grown on a mineral medium (Verduyn *et al.* 1992) at 30 °C at pH 5. The dilution rate was set to 0.1 h^{-1} and dissolved oxygen tension (DOT) was

maintained at over 30 % throughout the experiment. Consequently, the effect of oxygen limitation to xylitol production was attempted next. The oxygen limited conditions were created by sparging the bioreactor with mixture of air and nitrogen gas (5:95 %). As a consequence the DOT dropped down to a 0 % and the specific oxygen consumption (qO_2) and biomass concentration decreased by over 50 - 80 %. In addition, glucose was depleted and xylose, ethanol and glycerol accumulated compared to the corresponding aerobic conditions. However, no xylitol was produced (Table 4). Interpretation of these results was that *C. milleri* could not consume xylose under oxygen limited conditions, but the presence of varying amounts of glucose facilitated minor conversion of xylitol in fully aerobic conditions.

Table 4. Oxygen limited chemostat of *C. milleri* growing on a glucose-xylose mixture. No xylose was consumed or xylitol produced in these conditions.

D h^{-1}	Dry weight $g L^{-1}$	Glu(in) $g L^{-1}$	Xyl feed $g L^{-1}$	Xyl cons. $g L^{-1}$	Gly $g L^{-1}$	EtOH $g L^{-1}$	$q(O_2)$ mmol $g^{-1}h^{-1}$	Carbon recovery %
0.1	0.37	2.97	2.66	2.66	0.3	1.15	1.7	120

4.1.2 *In vitro* enzyme assays

The *in vitro* enzyme assays from glucose and glucose-xylose cultivations revealed that *C. milleri* does not posses XDH activity, which makes xylose utilisation for biomass synthesis unfeasible. The lack of XDH activity explains also, why the oxygen limited conditions did not induce xylitol production. However, a small, exclusively NADPH dependent XR activity (0.02 Umg^{-1}) was measured on a glucose–xylose mixture (as a comparison the XR activity of 0.82 ± 0.14 U mg^{-1} was found from *C. guilliermondii* from xylose grown cells). Consequently, a small xylitol flux was also measured (0.22 g L^{-1}) (Granström *et al.,* 2000). The cofactor dependency of aldehyde dehydrogenase (ALD) changed according to carbon substrate. On a glucose-xylose mixture only NADP-dependent ALD was active, whereas on a glucose ALD had a dual dependency for NAD(P) according to the *in vitro* enzyme assays. This indicates that to compensate for the increased requirement of xylose reductase for NADPH, *C.*

milleri does not increase the carbon flux into the pentose phosphate pathway (concluded from the enzyme assays of G6PDH), but generates NADPH through aldehyde dehydrogenase (Fig 3).

4.1.3 Metabolic flux analysis

The MFA that was constructed from the data of glucose and glucose-xylose steady states gave the tool to examine different scenarios and see how they can influence the flux distribution without influencing what we actually observe in terms of extracellular products. Furthermore, it allows to estimate the ATP yield and interpret the contribution of the maintenance energy and futile cycles to the ATP yield in different cultivation conditions. In the developed stoichiometric metabolic flux model three fluxes have been constrained: glucose uptake, flux to acetate and flux to cytosolic acetyl-CoA. Without constraint only 90 % of the measured glucose uptake was assimilated and major carbon flux was channelled through the PPP. In addition, acetate and cytosolic acetyl-CoA fluxes were negative and the ATP turnover was minimal.

When all the measured data from the glucose and glucose-xylose cultivations were applied into the constructed MFA the result indicated only 90 % consumption of the measured carbon source rate. Therefore, glucose uptake rate was constrained to use all the available glucose in both cultivations (glucose and glucose-xylose mixture). Consequently, the flux into PPP decreased to the level of 14 % and 9.8 % of the consumed glucose in glucose and glucose-xylose cultivations, respectively. This reduction suggests that flux through the PPP is decreased, when glucose uptake is increased according to the constructed metabolic network. When glucose uptake is

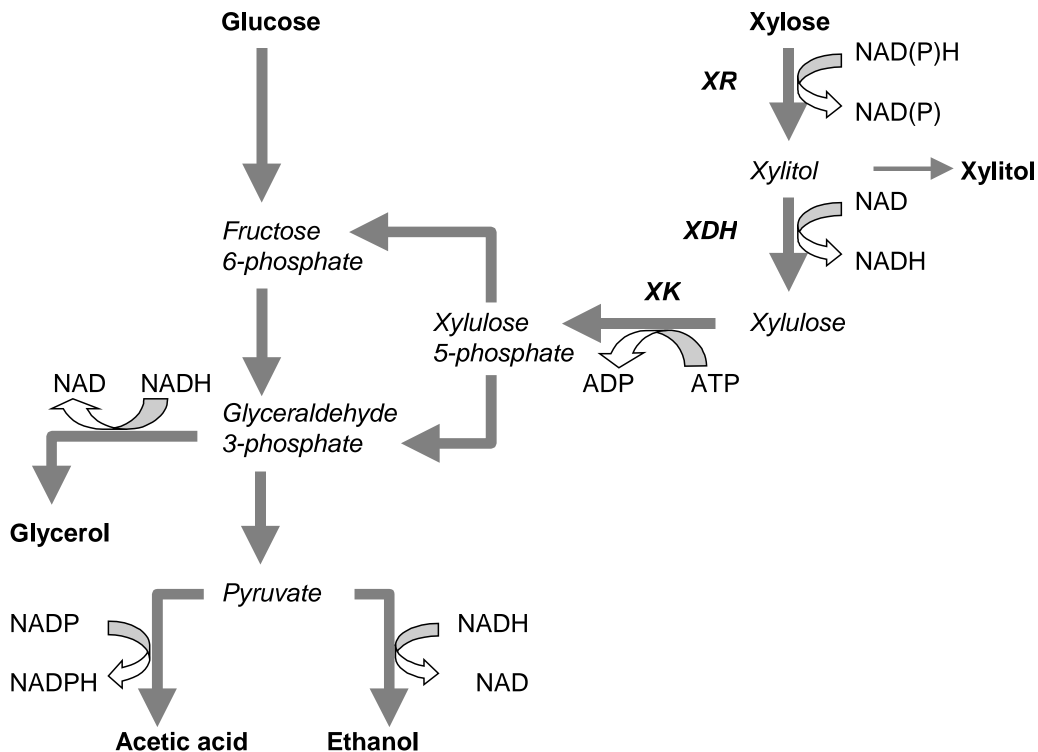


Fig 3. Metabolic pathways of glucose and xylose. Although *C. milleri* was able to produce 0.22 g L^{-1} of xylitol from xylose it could not grow in a xylose medium due to lack of XDH activity or lack of uptake mechanism for xylose. In a glucose-xylose medium only NADP-dependent ALD was active, whereas in glucose medium ALD had a dual dependency for NAD(P). The results suggested that NADPH for XR activity was generated through NADP-dependent ALD.

smaller the flux into PPP has to be increased in order to supply all the precursors for amino acids and NADPH for lipid synthesis. The used PO values for NADH (1.16) and FADH (0.1) are determined on the basis of having balance between consumption and production of ATP. However, this will not include the ATP consumption for maintenance or futile cycles. Therefore the Y_{XATP} values in glucose and glucose-xylose cultivations were significantly lower than the literature values. The reported Y_{XATP} values for NADH are close to 2 mol of ATP per C-mole of biomass (Geurts *et al.,* 1980). The Y_{XATP} values for glucose and glucose-xylose mixture were 0.28 and

0.22 mol ATP/C-mole CDW. When the PO value of 2 is used the Y_{XATP} increased to 1.1 mol ATP/C-mole CDW in both cultivations. The calculated results indicated that the presence of xylose decreased ATP yield (Granström *et al.,* 2000).

4.2 Xylitol production by *C. guilliermondii* (II)

The xylitol production characteristics of *C. guilliermondii* was studied in the shake flask and in the chemostat. Growth characteristics was studied in the chemostat in fully aerobic conditions and in the oxygen limited conditions. The correlation of *in vitro* enzyme activities and dilution rate was studied. No xylitol was produced in the chemostat. Xylitol production mechanism was demonstrated under transient oxygen limited condition.

4.2.1 Shake flask and chemostat experiments

The xylitol production capacity of *C. guilliermondii* was studied first in shake flask cultures on a complex medium. Complex medium was used to determine the highest xylitol yield and xylose consumption rates in optimal growth conditions. It was able to produce xylitol from xylose with a yield ($Y_{xol/xyl}$) of 0.61 g xylitol g xylose^{-1}. The biomass yield ($Y_{x/s}$) was 0.11 g CDW g xylose^{-1}. However, in a chemostat on a xylose medium under oxygen limitation, which was created by mixing nitrogen gas and air in ratio 5:1, *C. guilliermondii* produced no xylitol and only a small amount of glycerol (0.33 C-mM). This was accompanied with a reduction of biomass concentration and xylose accumulation.

C. guilliermondii was then cultivated in a chemostat with different dilution rates in fully aerobic conditions. It was found out that xylitol production from xylose is not growth related *ie*. xylitol is not produced due to increased growth rate (Fig 4). Therefore, a limited respiratory capacity cannot explain the xylitol production mechanism in *C. guilliermondii* as has been suggested for ethanol production from glucose in the long-term Crabtree effect in fully aerobic conditions in yeasts (Fiechter *et al*, 1981).

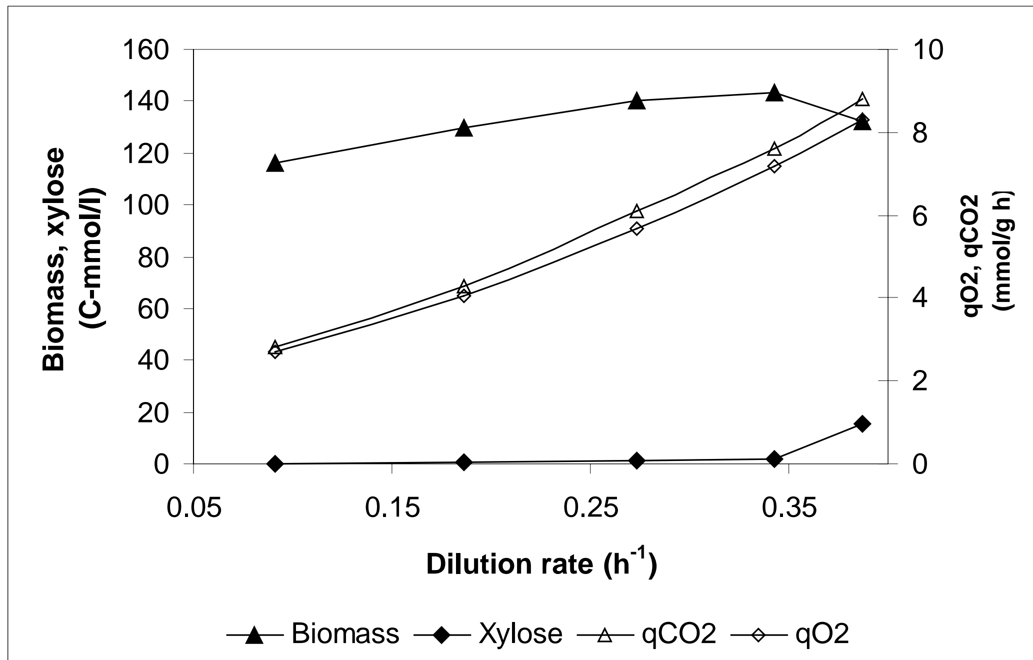


Fig 4. Chemostat cultivation of *Candida guilliermondii* on a mineral medium under fully aerobic conditions. The biomass, xylose, q_{O2} and q_{CO2} as a function of dilution rate. Steady state was established after five volume changes. Cultivation pH was 5.0 and temperature 30 °C. No other metabolite was detected than biomass and carbon dioxide. The maximum dilution rate (D_{max}) was determined according to the point, where xylose starts to accumulate in the fermenter.

4.2.2 Transient oxygen limitation

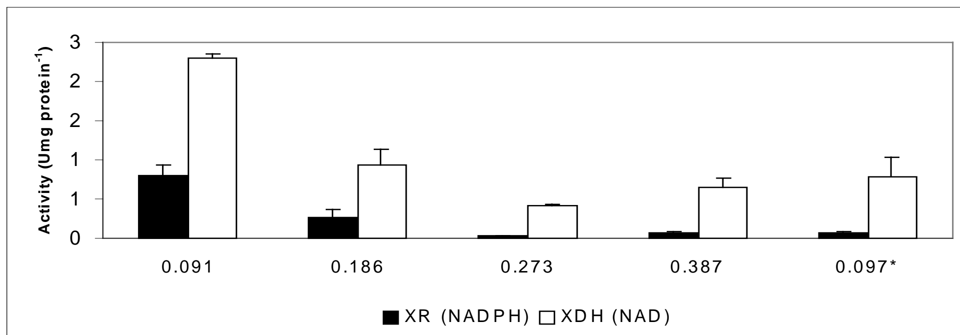
With a transient oxygen limitation method we were able to illustrate and confirm the xylitol production mechanism in *C. guilliermondii*. Transient oxygen limitation conditions were created by decreasing the oxygen transfer rate (OTR) steadily by decreasing the stirrer speed. This resulted in impaired xylose consumption and xylitol, acetate, glycerol and ethanol accumulation (Granström *et al.,* 2001). The intracellular accumulation of NADH is inhibiting XDH activity and this results in xylitol production (Oh *et al*, 1998; Hahn-Hägerdahl *et al.* 1994; Verdyun *et al.* 1985). The difference in metabolite production from xylose under constant and transient oxygen

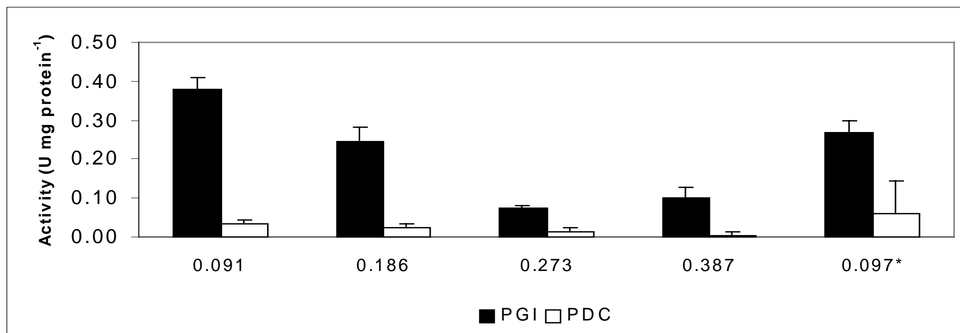
limitation indicates the effect of K_m and K_i values of different enzymes for NADH. This linear sequence may have a crucial role in metabolic regulation *ie.* in determining the uptake, fermentation or respiration of the substrate.

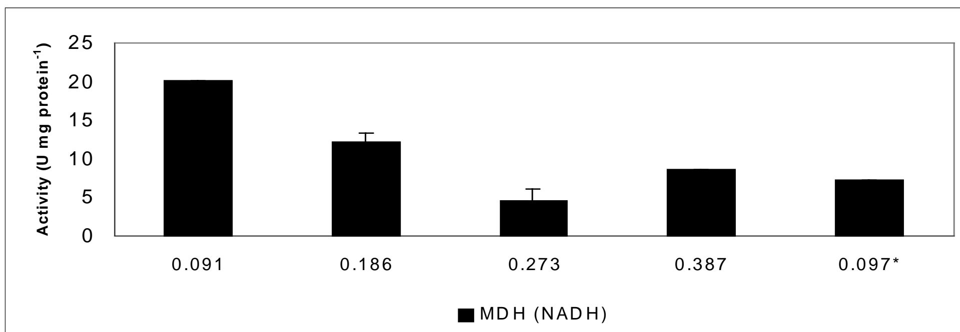
4.2.3 *In vitro* enzyme assays

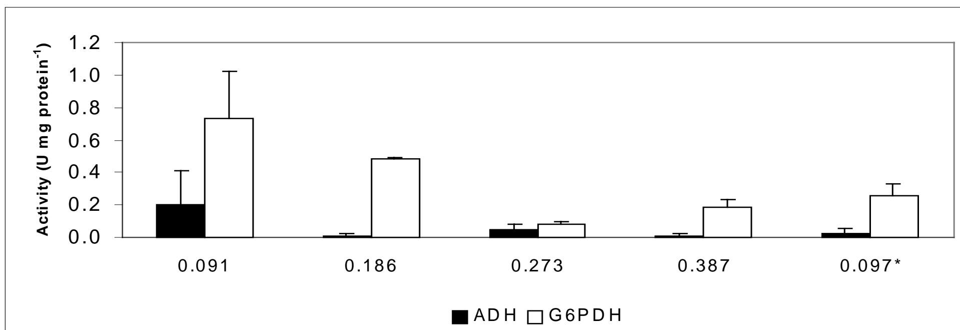
The *in vitro* enzyme assays were used to study xylose metabolism under different dilution rates in fully aerobic conditions (Fig 5). They showed that XR and XDH activities were higher with low dilution rates compared to the higher dilution rates. The same was also seen with phosphoglucoisomerase, malate dehydrogenase, NADP-dependent isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase. The only measured enzyme activity that clearly increased with increasing dilution rate was NAD-dependent isocitrate dehydrogenase, although a decline was observed at the dilution rate 0.186 h^{-1} (Fig. 5). Under constant oxygen limitation at the dilution rate 0.097 h^{-1} the XR and XDH activities were only 7.5 % and 34 % of those under fully aerobic conditions at similar dilution rate. The activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were 71 % and 35 % from those in fully aerobic conditions. The enzyme assays indicated that the activities of XR or XDH are not the limiting factors in xylose consumption. In addition, the NADP-dependent isocitrate dehydrogenase is not the main source of NADPH in these conditions. The measured activities of glucose 6-phosphate dehydrogenase and phosphoglucoisomerase indicate that substrates are cycled through PPP in order to regenerate cofactors (Granström *et al.,* 2001).

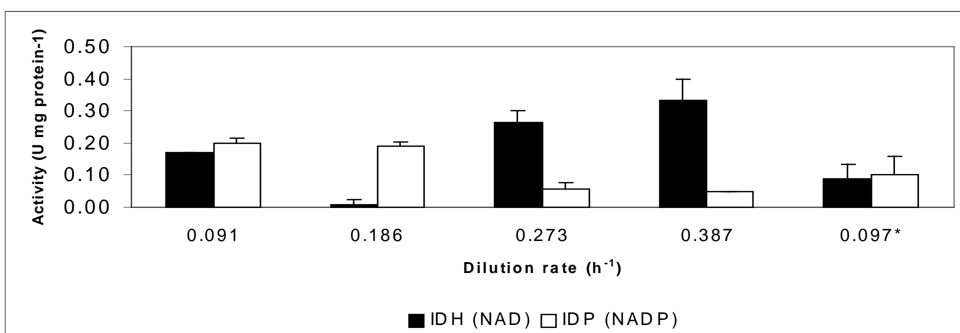
Fig 5. Summary of enzyme activities of *Candida guilliermondii* in the chemostat with xylose as the carbon substrate. Activities are expressed as U mg of protein^{-1} and results are given as mean ± standard deviation. All measurements were done in duplicate. Asterisk (*) stands for oxygen limited chemostat. XR = xylose reductase; PGI = phosphoglucoisomerase; PDC = pyruvate decarboxylase; ADH = alcohol dehydrogenase; IDH = NAD dependent isocitrate dehydrogenase; IDP = NADP dependent isocitrate dehydrogenase; G6PDH = glucose 6-phosphate dehydrogenase; XDH = xylitol dehydrogenase; MDH = malate dehydrogenase.


Activity (Umg protein-1)
3
2
2
1
1
0
0.091
0.186
0.273
0.387
0.097*
XR (NADPH)
XDH (NAD)


Activity (U mg protein-1)
0.50
0.40
0.30
0.20
0.10
0.00
0.091
0.186
0.273
0.387
0.097*
PGI
PDC


Activity (U mg protein-1)
25
20
15
10
5
0
0.091
0.186
0.273
0.387
0.097*
MDH (NADH)


Activity (U mg protein-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.091
0.186
0.273
0.387
0.097*
ADH
G6PDH


Activity (U mg protein-1)
0.50
0.40
0.30
0.20
0.10
0.00
0.091
0.186
0.273
0.387
0.097*
Dilution rate (h-1)
IDH (NAD)
IDP (NADP)

4.3 The effect of transient changes on metabolite production in *C. guilliermondii* and *C. tropicalis* (III).

The effect of different cofactor dependency of XR of *C. guilliermondii* and *C. tropicalis* on metabolite production pattern was demonstrated by novel method. This consisted of simultaneously decreasing agitation rate and increasing dilution rate. In addition, the response of these two yeasts to formate feed was studied in fully aerobic conditions.

4.3.1 The effect of linearly increasing dilution rate and decreasing agitation rate on metabolite production

We used linearly decreasing agitation rate and increased simultaneously the substrate feed in *C. tropicalis* and *C. guilliermondii*. The reasoning behind this experimental set up was to study the impact of different cofactor dependency of XR on metabolite production and xylose consumption under transient oxygen transfer rate (Granström and Leisola, 2002). Both strains accumulated xylitol, glycerol and xylose, but differed in ethanol and acetate accumulation. *C. tropicalis* accumulated ethanol but not acetate, whereas *C. guilliermondii* accumulated acetate, but not ethanol. Acetate production by *C. guilliermondii* indicates regeneration of NADPH through NADP-dependent acetaldehyde dehydrogenase. Although most of the NADPH is possible to generate through the pentose phosphate pathway (Bruinenberg *et al.,* 1985), acetate accumulation suggests that NADPH becomes a limiting factor for xylose conversion to xylitol in these conditions. In *C. tropicalis* NADPH limitation is partly compensated by NADH assimilation. This leads to higher xylose consumption and xylitol accumulation compared to *C. guilliermondii*. It is suggested that NADH is used for both converting xylose to xylitol and for inhibiting xylitol dehydrogenase in *C. tropicalis*. This difference in ethanol and acetate production is interpreted to be due to the differences in cofactor dependency of XR.

4.3.2 The effect of linearly increasing formate feed on metabolite production

When we had demonstrated that different cofactor dependency has definite effect on metabolite production pattern under transient changes we wanted to study how these two strains will respond to the linearly increasing intracellular NADH concentration in fully aerobic conditions. The fully aerobic conditions were chosen to maintain high xylose consumption rate. In addition, the combined effect of oxygen limitation and formate feed decreased the cell viability rapidly. Both strains were exposed to formate feed under similar conditions. Formate is catabolised into NADH and CO_2 by formate dehydrogenase enzyme in the cell. The steady state was established and formate feed profile was introduced in fully aerobic conditions. It was assumed that formate feed with accumulating intracellular NADH would lead to enhanced xylose utilisation and xylitol formation in *C. tropicalis* cultures due to the dual cofactor dependency of its XR but not in *C. guilliermondi*. Formate feeding exposed both yeasts to surplus of intracellular NADH, which was indicated by glycerol accumulation in fully aerobic conditions. *C. tropicalis* used the surplus of NADH as a cofactor together with NADPH as evidenced by enhanced specific xylose uptake. Due to exclusive NADPH dependency of the *C. guilliermondii* XR the effect of formate was different. Initially it facilitated slightly the specific xylose consumption, but had no effect on either xylitol or ethanol production (Granström and Leisola, 2002). These results indicated that *C. tropicalis* is more sensitive to accumulation of intracellular NADH by oxygen limitation. Based on this conclusion *C. tropicalis* was selected to be used in further xylitol production studies.

4.4 Purification of xylose reductase from *C. guilliermondii* (IV)

XR from *C. guilliermondii* was purified from a cell free extract by three consecutive steps using anion exchange, gel filtration and hydrophobic interaction chromatography. XR was the main protein in the cell free extract and its purity increased 17-times with the activity recovery of about 41 %. The estimated molar mass of the pure enzyme by SDS-PAGE was 36 000 g mol^{-1} (Fig. 6). The purified XR used only NADPH as a cofactor and had a pI value of 4.5 (Table 5). However,

some minor bands at pI 4.4 and 4.6 were detected, which indicates the presence of isoforms of the enzyme. XR displayed simple Michaelis-Menten kinetics. The Michaelis-Menten constants (k_{cat} and K_m) were determined (Granström *et al*, 2002a). The enzyme showed high affinity to L-arabinose, D-xylose and D-ribose, but a very low affinity to D-lyxose, L-xylose and L-ribose. *C. guillermondii* grew on all the tested pentoses. Growth with L-arabinose, L-ribulose, D-ribose and D-xylose was fast and with L-ribose, D-lyxose and L-lyxose somewhat slower.

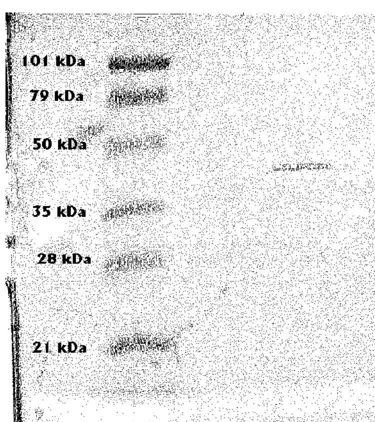


Fig 6. SDS-PAGE of *C. guilliermondii* xylose reductase obtained from various purification steps. First lane shows the mass standard. Third lane shows the molecular weight of the purified protein (Granström *et al*. 2002a).

Table 5. Comparison of different XR's purified from *Candida spp*.

Strain	Size (kDa)	pI	K_m (xylose) (mM)	Cofactor	Reference
C.tropicalis	36	4.15	37	NADPH	Yokoyama *et al.* 1995
C.tenuis	43	4.7	72	NAD(P)H	Neuhauser *et al.* 1997
C.guilliermondii	36	4.5	79	NADPH	Granström *et al.* 2002

4.5 Xylitol production by *C. tropicalis* (V)

The growth characteristics of *C. tropicalis* was studied in the xylose limited chemostat on mineral medium in fully aerobic conditions. Next, oxygen limited chemostat was established in order to obtain constant xylitol production rate. The effect of formate feed on the metabolite production was studied. *In vitro* enzyme activities were studied in these conditions. MFA was carried out to demonstrate the possible flux distribution and the effect of substrate cycling on ATP yield.

4.5.1 Chemostat experiments

Similarly to *C. guilliermondii* the xylitol production was not growth related in *C. tropicalis* (Fig 7). At dilution rates higher than 0.35 h^{-1} biomass and gas values showed tendency to oscillate, but still all the xylose was consumed. The maximum dilution rate could not be determined accurately due to the unstable conditions at the dilution above 0.4 h^{-1}. No xylitol was produced in these fully aerobic conditions.

4.5.2 Method for establishing an oxygen limited chemostat

The oxygen limited steady states where the xylitol production rate was highest were established. Initially, this was determined by simultaneously changing the agitation profile from 2000 rpm to 100 rpm and the dilution rate profile from 0.1 h^{-1} to 0.4 h^{-1} during 10 h (Fig 8). The key metabolites were measured once an hour. The conditions prevailing under the transient changes could be reproduced in the chemostat by terminating both profiles at a desired time point and allowing the cultures to achieve a steady state. This was done at the time point, where the xylitol production rate was the highest. In these conditions also ethanol and glycerol were produced and residual xylose accumulated confirming the oxygen limitation. Two separate oxygen limited steady states carried out using these profiles gave following mean values for specific xylose consumption, $q(CO_2)$ and $q(O_2)$ respectively: 774 ± 26 C-mmol C-mol CDW^{-1} h^{-1}, 246 ± 12 C-mmol C-mol CDW^{-1} h^{-1} and 202 ± 4.8 mmol C-mol CDW^{-1} h^{-1}. This indicates that the method for establishing the oxygen limited chemostat is repeatable.

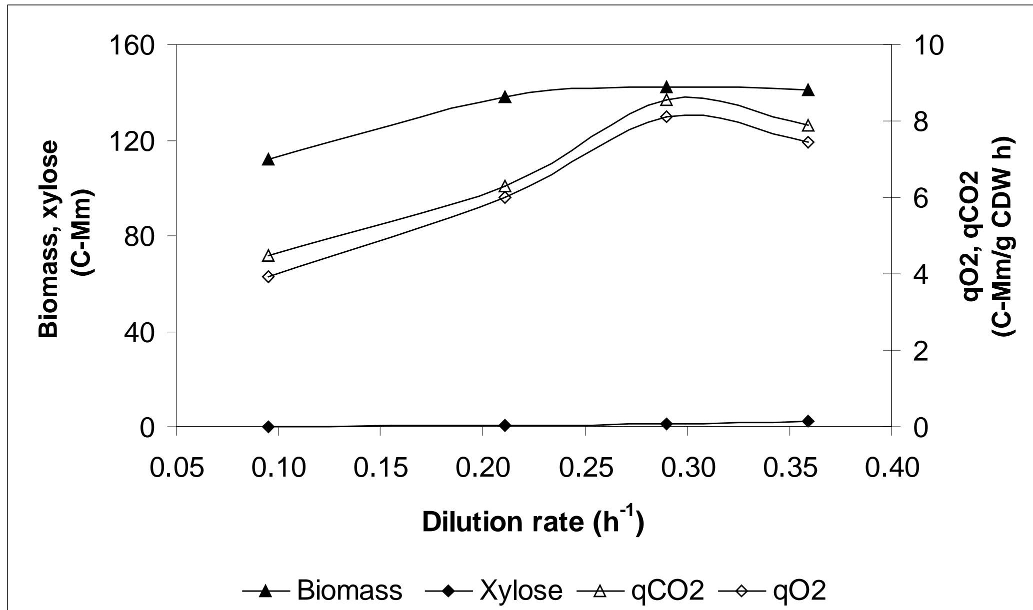
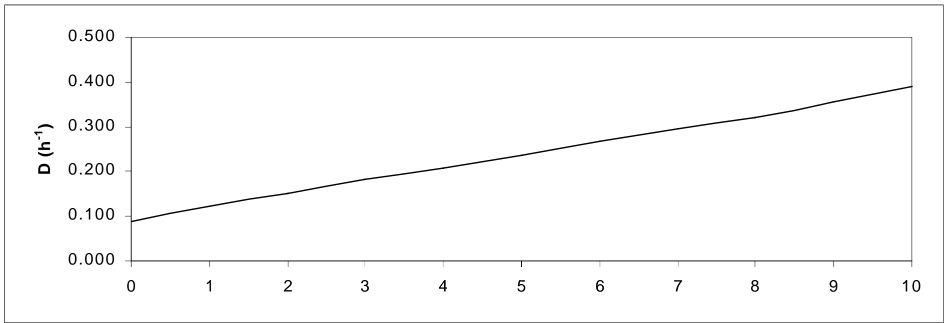
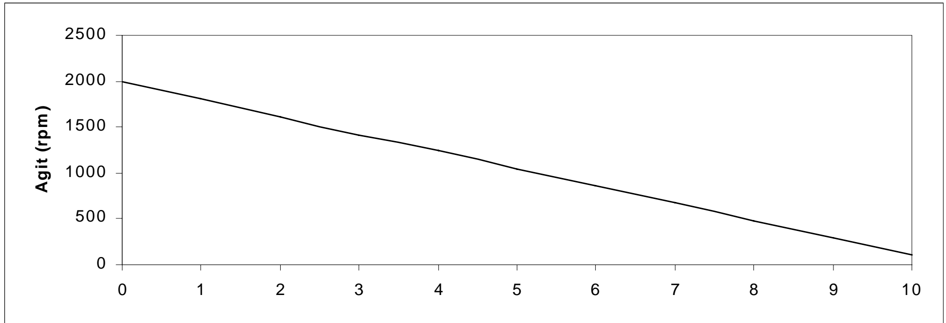
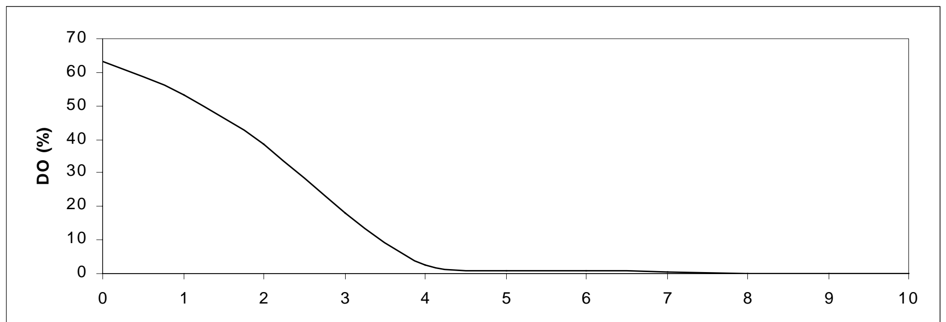


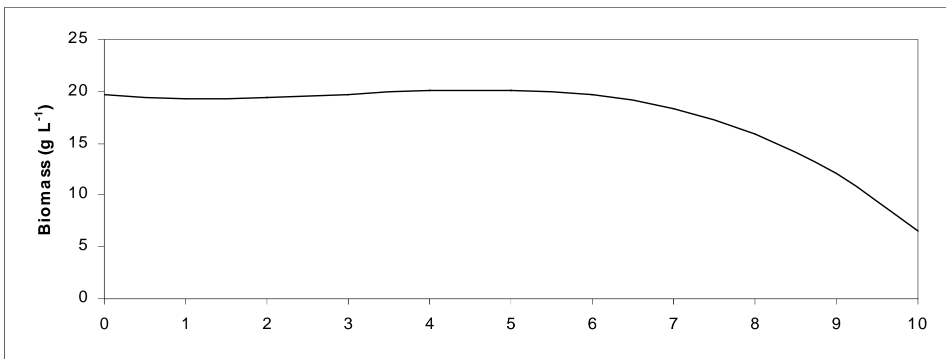
Fig 7. Chemostat cultivation of *C. tropicalis* on a mineral medium with xylose limitation. Biomass, xylose, q_{O2} and q_{CO2} are presented as a function of dilution rate. Steady state was established after five volume changes. Cultivation pH was set to 5.0 and temperature to 30 °C. No other metabolites than biomass and carbon dioxide were produced. The onset of xylose accumulation occurred after the dilution rate was increased to 0.35 h^{-1}.

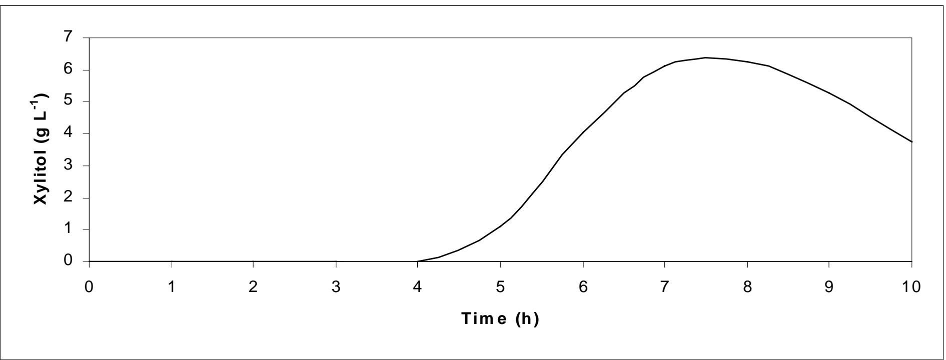
Fig 8. Transient changes of *C. tropicalis* was studied in establishing an oxygen limited chemostat. Dilution rate, agitation rate, dissolved oxygen, biomass concentration and xylitol concentration was followed and measured hourly. The xylose concentration in the feed was 50 g L^{-1}. These gradients were applied and they were stopped at the 6. hour. In these conditions xylitol flux was increasing, but xylose was not yet accumulating in high amounts (0.28 g L^{-1}). The values of dilution rate, agitation rate, dissolved oxygen was chosen for the growth parameters of the oxygen limited chemostat. The cultivation was let to adapt into these growth conditions. After five volume changes an oxygen limited steady state was reached. Data is taken from Granström and Leisola (2002).


0.500
0.400
0.300
0.200
0.100
0.000
D (h-1)
0 1 2 3 4 5 6 7 8 9 10


2500
2000
1500
1000
500
0
Agit (rpm)
0 1 2 3 4 5 6 7 8 9 10


70
60
50
40
30
20
10
0
DO (%)
0 1 2 3 4 5 6 7 8 9 10


25
20
15
10
5
0
Biomass (g L-1)
0 1 2 3 4 5 6 7 8 9 10


7
6
5
4
3
2
1
0
Xylitol (g L-1)
0 1 2 3 4 5 6 7 8 9 10
Time (h)

The transient method applied in this work represents a novel application of continuous culture. Kask *et al.* (1999), Müller *et al.* (1985) and Paalme *et al*. (1997) have used a similar approach before, but varied only one parameter at a time. This method can be used routinely for screening of growth characteristics of potential host organism. We have already studied the physiology of *C. tropicalis* under transient conditions (Granström and Leisola, 2002). Consequently, we wanted to establish a steady state to study the combined effect of substrate feed, oxygen limitation and formate feed to xylitol production in the chemostat. The steady state conditions was also prerequisite for metabolic modelling by MFA.

4.5.3 Oxygen limited chemostat and the effect of formate cofeeding

An oxygen limited chemostat was created using the method described above (Granström *et al*. 2002b). The constant specific xylitol production rate q(xol) 149 C-mmol xylitol C-mol CDW^{-1} h^{-1} (0.169 g xylitol g CDW^{-1} h^{-1}) was obtained at the steady state (Table 6). Also, ethanol and glycerol was produced confirming the oxygen limited conditions. Consequently, the effect of formate feed on xylitol production was studied at oxygen limited steady state conditions. Formate catabolism results in CO_2 and NADH by the action of formate dehydrogenase. In this case formate was used to increase the intracellular concentration of NADH. We assumed that excess NADH would result in higher oxygen and xylose consumption and correspondingly increase xylitol production by inhibiting XDH enzyme. Very low formate cofeeding was introduced and culture was let to reach the steady state. The formate cofeeding increased the q(xol) by 7 % to the value of 160 C-mmol xylitol C-mol CDW^{-1} h^{-1} (0.189 g g CDW^{-1} h^{-1}). In addition, specific xylose and oxygen consumption rates as well as ethanol and CO_2 production rates increased (Table 6).

Table 6. Consumption and production rates by *Candida tropicalis* in the oxygen limited chemostat under high xylose concentration. The cultivations were done at 30 °C on a mineral medium at pH 5. The dilution rate for xylose was 0.27 h^{-1} in both cultivations and for cosubstrate formate it was 0.0012 h^{-1} in xylose-formate cultivation.

Run	Xylose	Biom	Formate	q(O2)	q(CO2)	Xylitol	Glycerol	EtOH	Carbon balance
	C-mmol C-mol CDW^{-1} h^{-1}								
Xylose control	774	268	-	202	246	149	3.4	81	98
Xylose-Forma te	842	274	4.8	231	271	160	4.8	99	98

4.5.4 *In vitro* enzyme assays of oxygen limited chemostat growing on xylose and xylose-formate

The enzyme assays indicated that XR of *C. tropicalis* is mainly NADPH dependent, but a small and consistent NADH dependent activity was also found from xylose and xylose-formate cultivations (Table 2 in the article V) Glucose 6-phosphate dehydrogenase activity was found from both cultivations suggesting that NADPH was, at least, partly generated through PPP. Both NAD and NADP dependent activities of isocitrate dehydrogenase were found, but not the malate synthase activity. This indicates that glyoxylate cycle was not active under these conditions, whereas TCA cycle was (Granström *et al* , 2002b). Malic enzyme was found to be active with both NADH and NADPH, whereas malate dehydrogenase was exclusively NAD-dependent. Expression of both of these enzymes at the same time enables substrate cycling, where NADPH can be converted to NADH or *vice versa* according to the *in vivo* metabolism of the *C. tropicalis* (Fig 9). Only traces of phosphoglucoisomerase,

pyruvate kinase, phospho-*enol*-pyruvate carboxykinase and fructose 1,6-bisphosphatase activities were detected from xylose and xylose-formate oxygen limited cultivations (results not shown). However, phosphoglucoisomerase should be active in order to synthesise storage carbohydrates from xylose. Low activities of pyruvate carboxylase, pyruvate decarboxylase, alcohol dehydrogenase and aldehyde dehydrogenase were found from both cultivations. The expression of PK, PYC and PEPCK simultaneously maintains the ATP turnover under these oxygen limited conditions (Fig. 9). The enzyme assays indicated that one possibility for *C. tropicalis* to maintain the ATP turnover and redox balance under oxygen limited condition is to cycle these substrates.

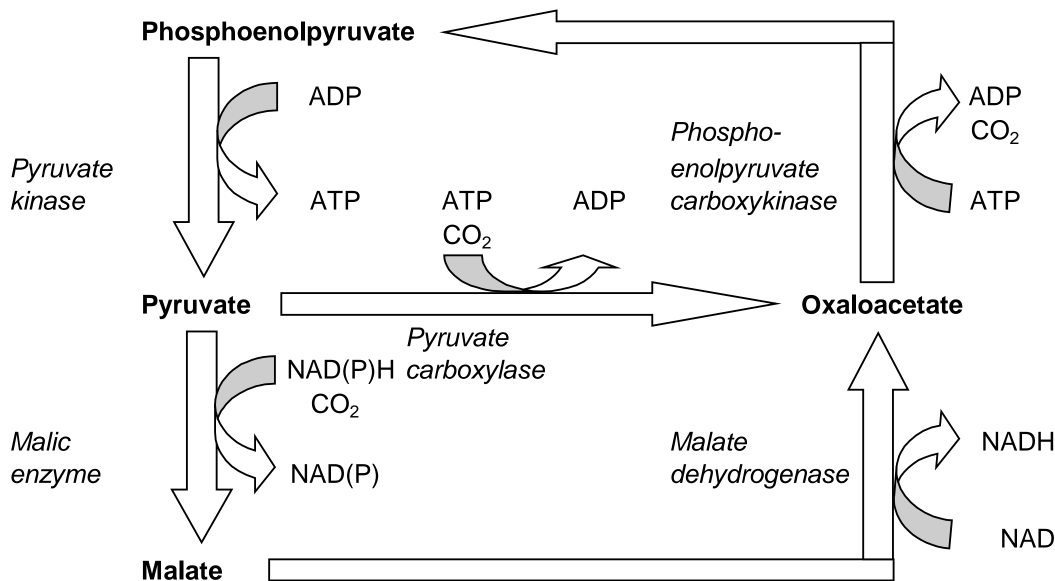


Fig 9. Simultaneous expression of pyruvate kinase (PK), pyruvate carboxylase (PYC) and phospho-*enol*-pyruvate carboxykinase (PEPCK) enables substrate cycling in order to maintain the ATP turnover. Similarly, when malic enzyme (ME) and malate dehydrogenase (MDH) are expressed at the same time a transhydrogenase cycle is created, where NADPH is converted to NADH. Also, ME can function as a redox sink for cytosolic NADH. Malate allows an alternative way for pyruvate to cross the mitochondrial membrane.

4.5.5 Metabolic flux analysis of oxygen limited chemostat

The MFA was based on the earlier published model in the article I (Granström *et al.,* 2000). In the present model we have further emphasised substrate cycling between cytosol and mitochondria. This approach enabled us to study the effects of different metabolic pathways implicated by *in vitro* enzyme assays on ATP yield in xylose and xylose-formate cultivations. The oxygen limited xylose cultivation model contained 99 compounds and 85 reactions and the xylose-formate cultivation contained 101 compounds and 87 pathway reactions. It indicated that the flux into pentose phosphate pathway (PPP), the upper part of the glycolytic flux and the gluconeogenetic flux were increased, whereas the TCA cycle fluxes from acetyl CoA to succinate is markedly reduced in the xylose-formate cultivation. Consequently, fructose 6-phosphate is replenished from PPP in sufficient amount for synthesis of storage carbohydrates and generation of NADPH through glucose 6-phosphate dehydrogenase. The fructose 1,6bisphosphatase flux is close to zero. The malic enzyme has a negative flux resulting in malate instead of pyruvate. The MFA combined with *in vitro* enzyme assays demonstrated three intracellular substrate cycles between 1) pyruvate carboxylase – pyruvate kinase – phospho-*enol*-pyruvate carboxykinase; 2) malate dehydrogenase – malic enzyme; 3) cytosolic and mitochondrial alcohol dehydrogenase - acetaldehyde dehydrogenase. The measured activities of malate dehydrogenase, malic enzyme and pyruvate carboxylase were consistent with the model, but the results of pyruvate kinase and phospho-*enol*-pyruvate carboxykinase enzyme assays cannot be interpreted as straightforwardly.

In order to substantiate the results of the *in vitro* enzyme assays the fluxes of phosphoglucoisomerase, pyruvate kinase, fructose 1,6-bisphosphatase and phospho-*enol*-pyruvate carboxykinase were constrained to zero one at a time from the metabolic network (Granström *et al.,* 2002b) The numerical solution of constrained linear least-squares (CONLS) method by MATLAB was found. Consequently, all the ATP producing reaction rates were summed up and ATP yield was calculated (Table 7). The results indicated that if pyruvate kinase or phospho-*enol*-pyruvate carboxykinase fluxes are constrained to zero the ATP yield is reduced by approximately 60 % in both cultivations. This was considered as a non-feasible

solution. The highest ATP yield was obtained, when fructose 1,6-bisphosphatase flux was constrained to zero in both cultivations. Theoretically, this is a feasible solution and it is according to the measured results: only traces of fructose 1,6-bisphosphatase activity was found. This would mean that under these conditions the xylose metabolism of *C. tropicalis* would resemble the "tuning fork" model (see fig 4). In the tuning fork model xylose is provided from PPP to fructose 6-phosphate and glyceraldehyde 3-phosphate. Fructose 6-phosphate is directed to glucose 6-phosphate and further on to storage carbohydrates and PPP. Whereas, glyceraldehyde 3-phosphate is directed to glycolytic pathway. However, if all these enzymes are expressed and active under the oxygen limited conditions the ATP yield of xylose and xylose-formate cultivations is 6.9 and 8.7 mol ATP/C-mol CDW respectively (Table 8). The increase in ATP yield in xylose-formate cultivation is due to the addition of NADH from formate catabolism.

Table 8. ATP yields of xylose and xylose-formate cultivations calculated by metabolic flux analysis (MFA). Different simulations were carried out by constraining phosphoglucoisomerase (PGI), pyruvate kinase (PK), fructose 1,6-bisphosphatase (FBPase) and phospho-*enol*-pyruvate carboxykinase (PEPCK) fluxes to zero one at a time from the metabolic network. Control refers to the cultivation where all the PGI, PK, FBPase and PEPCK were all active.

Cultivation	Y_{xATP} (mol ATP / C-mol CDW)				
	Control	PGI=0	PK=0	FBPase=0	PEPCK=0
Xylose	6.8	4.1	2.2	7.0	2.9
Xylose-formate	8.0	5.7	2.4	8.3	3.3

4.6 Semi-continuous-resting-cell-method (SCREM)

4.6.1 Background of the SCREM method

After the physiology studies with three different *Candida* strains we were able to determine the best possible cultivation method and strain for xylitol production studies. *C. tropicalis* was decided to use in this experiment due to its dual dependent XR for NADH and NADPH. This allows more sensitive response to oxygen limitation than exclusively NADPH dependent XR (Granström and Leisola, 2002). The main goal of this experiment was to achieve an industrially acceptable xylitol yield from xylose.

We had earlier found out that xylitol production is not dependent on growth rate with *C. tropicalis*. Therefore, the reduction potential of the non-growing yeast biomass was decided to use a catalyst for xylose reduction under oxygen limited conditions. After a number of trials an modified repeated fed batch method was developed. This was particularly suitable for *C. tropicalis* for three reasons: 1) xylose is taken up by passive diffusion, 2) it has very high specific xylose consumption rate 3) it tolerates xylose concentration up 120 g L^{-1} (Ojamo, 1994).

4.6.2 Experimental set up

Yeast cells were first grown in a chemostat mode (see Materials and Method section 3.2) up to the cell dry weight of 53.3 g L^{-1} on a mineral medium with 100 g L^{-1} of xylose as a substrate. Working volume of the bioreactor was 1.5 L. At the steady state the yeast suspension was concentrated down to 0.3 - 0.4 L with ultrafiltration (Pellicon 2, 0.1 m^2 Millipore Corp., USA) in order to increase the cell dry weight between 200 - 250 g L^{-1}. The dissolved oxygen was maintained at 0 % by agitation (Fig 11). This inhibited the cell growth, but sustained the reduction potential of the yeast cell mass. Under these conditions xylose solution was pumped into the bioreactor increasing the concentration of xylose approximately to 100 g L^{-1}. Most of the xylose was reduced to xylitol, but also ethanol, glycerol and acetate was produced. After the most effective xylitol production phase (2-8 h), the agitation rate was

increased and the working volume of the bioreactor was increased to 1.5 L again. The cells were let to regenerate overnight. Next day the production phase was repeated. On a two successive experiments the initial biomass concentration was 45.3 g L^{-1} and 53.3 g L^{-1}. The maximum volumetric productivity achieved was 5.7 g xylitol L^{-1} h^{-1} with the yield of 69 % (Fig 10 and 11).

This experiment indicated that yeast cells could maintain their reduction potential without significant biomass growth (resting cells). However, due to the high concentration of biomass the specific xylitol production is very small (0.06 g xylitol g

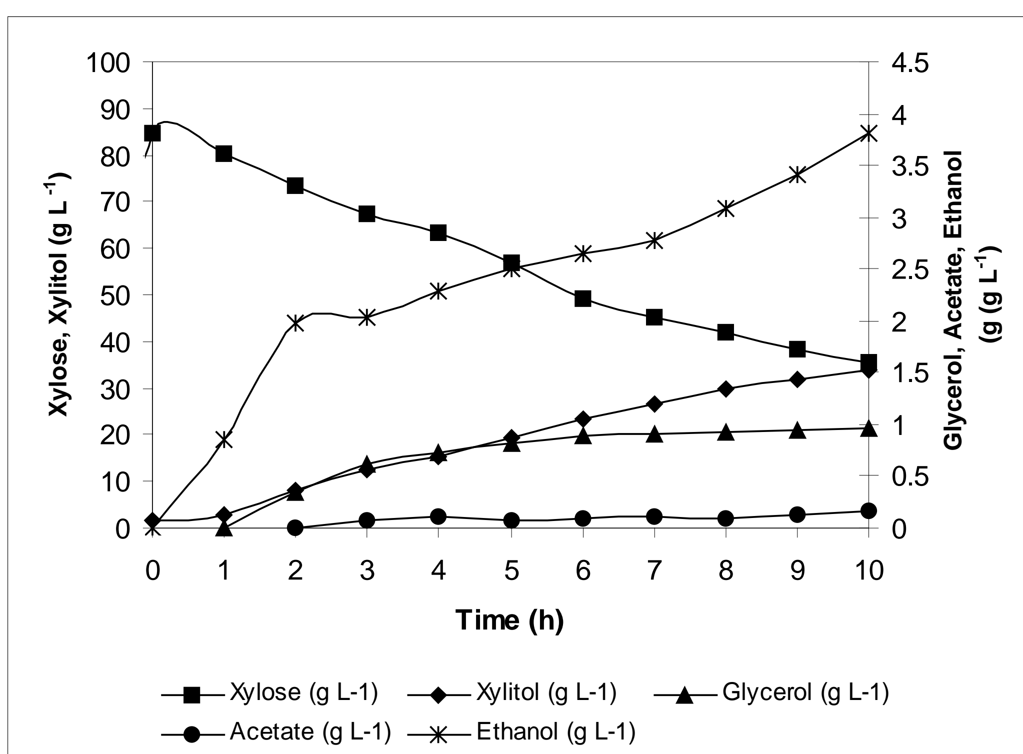


Fig 10. Xylitol production with *C. tropicalis* using a Semi-Continuous-Resting-Cell-Method (SCREM). The cells were initially grown in a chemostat mode. Before the production phase the cell suspension was ultrafiltrated in order to obtain a high cell density. Xylose solution was pumped into the bioreactor giving the final xylose concentration close to 100 g L^{-1}. Oxygen level was controlled by agitation.

CDW^{-1} h^{-1}). The advantage of using this method, as opposed to batch process or fed batch, is that SCREM is semi-continuous and it can be fully automated. The level of reduction capacity (*ie.* cell viability) can be monitored by pH and dissolved oxygen level control. Dissolved oxygen level has considerable effect on volumetric productivity of xylitol (Fig 11). Initially volumetric productivity increases close to 8 g xylitol L^{-1} h^{-1}, but decreases after 4 hour due to increase in dissolved oxygen level. In addition, biomass begins to grow. This indicates the importance of dissolved oxygen control in this modified repeated fed batch production method. If dissolved oxygen level or pH deviates extensively during production phase, it will result in cell lysis and prevents xylose reduction. The average yield between 2 - 8 hours is 0.69 g xylitol g xylose^{-1}. When the yield values are calculated hourly, also higher values are obtained (Fig 11).

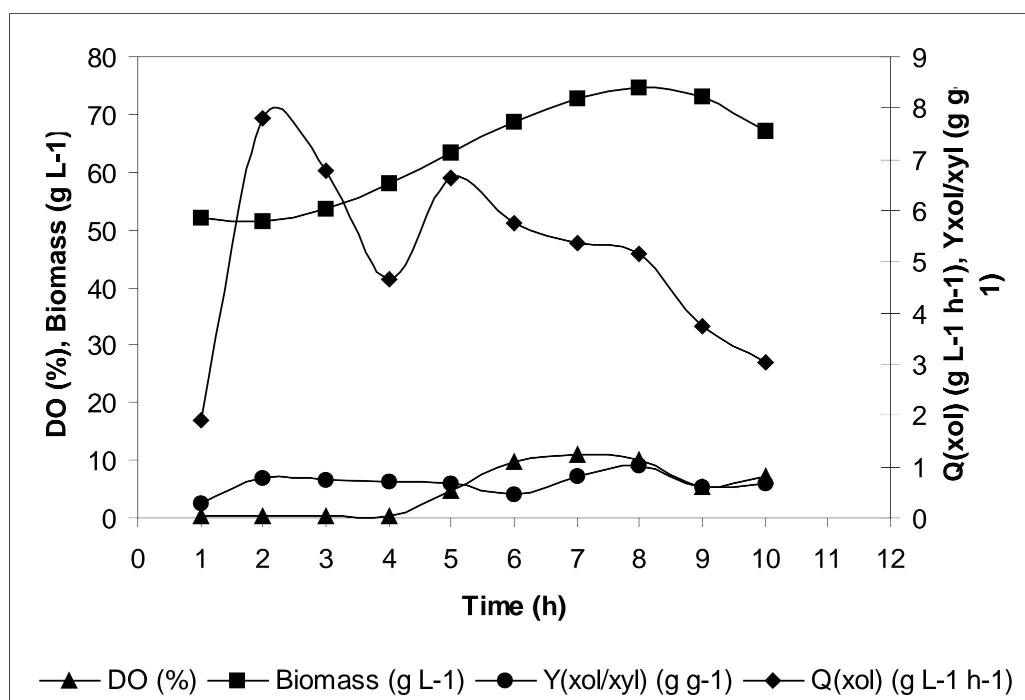


Fig 11. The Semi-Continuous-Resting-Cell-Method (SCREM) used for xylitol production with *Candida tropicalis*. The average volumetric productivity value between 2 - 8 hours was 5.7 g xylitol L^{-1} h^{-1} and the yield of xylitol was 69 %. All the xylitol production studies were done on a mineral medium at pH 5 at temperature 30 °C.

5 DISCUSSION

The main goal of this work was to developed industrially profitable xylitol production method. Three different *Candida* yeasts *ie*. *C. milleri, C. guilliermondii* and *C. tropicalis* were used as model organisms. Their xylose metabolism was studied by chemostat cultivation and *in vitro* enzyme assays. Their metabolism was modelled with computer simulation using metabolic flux analysis. *C. tropicalis* was selected to be used as a candidate strain for production studies due to its double dependent XR for NADH and NADPH. This facilitates its xylitol production characteristics in the bioreactor under oxygen limited conditions.

The new method for xylitol production was modified from repeated fed batch type cultivation. It was named as SCREM (semi-continuous-resting-cell-method). Using this method the average volumetric productivity of 5.7 g xylitol $L^{-1} h^{-1}$ was achieved with the 69 % yield of xylitol from xylose on a mineral medium. Higher xylitol yields from xylose have been reported by number of researchers, for example Meyrial *et al.* (1991) and Choi *et al.* (2001). Addition of yeast extract or cosubstrate will have an immediate effect on xylitol production (Horitsu *et al.* 1992; Yahashi *et al.,* 1996). However, we have achieved the highest volumetric productivity hitherto on a strictly mineral medium with xylose as the sole carbon and energy source using this new bioprocess set up. Yeast suspension can be regenerated multiple times similar to the breweries. In our case we had one production phase per day and yeast cells were let to regenerate overnight. We run this bioprocess set up for one week, but different trials with formate feeding were carried out each day. Therefore, only the results of the highest xylitol productivity is reported here.

The physiology of *C. tropicalis* under oxygen limitation was studied in the chemostat. The *in vitro* enzyme assays indicated that the enzyme activities, which allows substrate cycling between PK - PYC - PEPCK and MDH - ME takes place to maintain the sufficient ATP turnover and redox balance under oxygen limited conditions. Under these conditions the normal enzyme regulation does not necessarily occur. Thus the enzymes of glycolytic pathway may operate simultaneously with the

enzymes of gluconeogenetic pathway. In respect to xylitol production this means greater challenge *eg*. for metabolic engineering. According to Stryer (1995) one possibility for these substrate cycles (also called futile cycles) is amplification of metabolic signals. Another possibility is the reoxidation of NADH through shuttling of substrate between different compartments (Bakker *et al.,* 2001). Aristidou *et al.* (2000) have shown that the expression of NAD- and NADP-linked glutamate dehydrogenases and a cycle comprising NADP-linked malic enzyme, pyruvate carboxylase and NAD-linked malate dehydrogenase can increase the yield on carbon source of desired products such as ethanol and the specific rate of product formation.

In our case we have modelled the effect of these proposed cycles on ATP yield and redox balance.The MFA was constructed in order to theoretically study the intracellular fluxes of *C. tropicalis* in oxygen limited chemostat. Three different intracellular substrate cycles were modelled between 1) pyruvate carboxylase - pyruvate kinase - phospho-*enol*-pyruvate carboxykinase, 2) malate dehydrogenase–malic enzyme and 3) cytosolic and mitochondrial alcohol dehydrogenase - aldehyde dehydrogenase. The function of these substrate cycles is to maintain sufficient ATP turnover, transhydrogenase activity between NADH - NADPH and to maintain the redox balance between cytosol and mitochondria, respectively. Also, MFA was used to interpret the results from *in vitro* enzyme assays of *C. tropicalis* in oxygen limited chemostat. The measured enzyme activities of malate dehydrogenase, malic enzyme and pyruvate carboxylase were consistent with the model, but the results of pyruvate kinase and phospho-*enol*-pyruvate carboxykinase enzyme assays cannot be interpreted as straightforwardly. The ATP yield calculation by MFA was used to justify the presence of pyruvate kinase and phospho-*enol*-pyruvate carboxykinase. Excluding these fluxes from the metabolic network decreased the ATP yield with almost 60 %.

When choosing between the studied *Candida spp*, which strain will be used for xylitol production studies *C. guilliermondii* and *C. tropicalis* were compared. These were selected based on their different cofactor dependency of XR for NADH and NADPH. XR of *C. tropicalis* has dual a dependency, whereas XR of *C. guilliermondii* is only

dependent on NADPH. Using a novel research method of ascending and descending profile feeding we were able to demonstrate the differences between the extracellular metabolite production and cofactor dependency of XR. *C. guilliermondii*, which XR is exclusively NADPH dependent, accumulated acetate in order to regenerate NADPH. Conversely, *C. tropicalis*, which XR is partly NADH dependent, accumulated ethanol in order to regenerate NADH. In addition, glycerol acts as a redox sink in both cases (Oura, 1977).

When we had found out that different cofactor dependency has different response to metabolite production, we wanted to study, what kind of effect has additional NADH for xylitol flux? This was done by formate cofeeding. Formate is catabolised to NADH and CO_2 in the cell. The exclusively NADPH dependent XR of *C. guilliermondii* accumulated glycerol, xylose and formate. It was considerably more tolerant to the effect of formate than partly NADH dependent XR of *C. tropicalis*. The formate feeding resulted in higher xylose consumption and xylitol, ethanol and glycerol production, but did not increase the biomass production in *C. tropicalis*. As a consequence *C. tropicalis* was much more sensitive to additional NADH emerging from formate catabolism. Therefore, it was selected to be used in xylitol production studies. In addition, this shows that controlling the redox balance of yeasts it is possible to regulate the xylose uptake and metabolite production to some extent. This is particularly significant in situations, where xylose uptake occurs by facilitated diffusion.

C. guilliermondii was able to produce xylitol from xylose with the yield of 61 % in the shake flask on a complex medium. In the chemostat under oxygen limitation xylitol was not produced. Oxygen limitation resulted in biomass washout, xylose accumulation and glycerol production. Why xylitol was produced in the shake flask, but not under oxygen limitation in the chemostat? The *in vitro* enzyme assays indicated that both XR and XDH are active under these conditions. The amount of XR or XDH protein is not rate limiting, because the highest enzyme activities were measured with the slowest dilution rate. Also, under oxygen limitation the XR and

XDH activities had approximately the same values as with fully aerated conditions with a dilution rate of 0.3 h^{-1}.

Introducing an agitation profile at steady state conditions solved this question. This decreased the oxygen transfer rate linearly and resulted in xylose accumulation and xylitol, acetate, glycerol and ethanol production. The interpretation of this result was that the amount of intracellular NADH increased due to inadequate respiration causing the accumulation of these redox metabolites. It is suggested that the onset of the accumulation of the metabolites happened in a sequence determined by different K_m and K_i values of these proteins for NADH. By adjusting the oxygen level with high accuracy it could be possible to control the order of accumulation of these metabolites, also in the chemostat. In the shake flask, slow biomass growth combined with oxygen limitation ensures that most of the carbon flux goes into xylitol and only the necessary carbon flux is directed into PPP. The same yield was achieved with *C. tropicalis* in the xylitol production studies on a mineral medium. The *in vitro* enzyme activities suggested that substrate cycling enables the cofactor regeneration through phosphoglucoisomerase and glucose 6-phosphate dehydrogenase.

The purification of XR from *C. guilliermondii* showed that all the common and also rare pentoses are utilised by this yeast. D- and L-forms of pentoses were reduced to their respective polyalcohols by pure protein. In case of using lignocellulose as a raw material for biotechnological production of ethanol this is an advantage, which allows utilisation of all pentose sugars. In the case of xylitol production this is, however problematic since the different polyols produced are difficult to separate from each other. However, in the future, those enzymes, which have more selective characteristics may become even more important.

Xylitol production from xylose by *C. milleri* was negligible. Cofeeding of glucose did not facilitate the uptake of xylose in the chemostat. One reason for this can be the lack of sufficient amount of XR protein since the measured activity was negligible. However, the change in the NAD/NADP ratio of *in vitro* activity of aldehyde dehydrogenase (ALD) suggested that cofactor regeneration could be one of the

bottlenecks in xylose reduction to xylitol. Oxygen limitation did not result in xylitol production. This is propably due to the lack of XDH in xylose catabolic pathway in *C. milleri.* For the same reason it is impossible to use xylose for biomass synthesis by *C. milleri*. It can be speculated that the presence of glucose repressed the activity of XDH, which was not found from the cell free extracts of *C. millerii.* However, in the bioreactor cultivation the glucose was always depleted, whereas xylose was always present.

The constructed MFA indicated that flux into PPP increased due to the presence of xylose. The MFA pointed out that one of the PPP metabolites, ribulose-5-phosphate, becomes limiting in xylitol production. Ribulose-5-phosphate is the precursor of 5-phosphoribosyl pyrophosphate (PRPP). The MFA indicated that this flux is negative *ie.* ribulose-5-phosphate is synthesised. This result was quite intruiging (nonetheless irrelevant in respect to industrial xylitol production) due to the fact that the level of PRPP is increased in humans having xylitol in their daily diet (Mäkinen, 2001). However, when interpreting this result we cannot underestimate the complexity of cell metabolism.

6 CONCLUSIONS AND FUTURE CONSIDERATION

Yeasts convert xylose into xylitol by one enzymatic step. It is concluded that subsequent inhibition of xylitol dehydrogenase enzyme by intracellular NADH will redistribute xylitol out of the cell efficiently. It is questionable whether this type of biotechnological process will never overcome the chemical hydrogenation in xylitol production allthough the possibility to use crude industrial sidestreams is one the advantages of the biotechnological production method over the chemical reduction. On the other hand simple one-step enzymatic conversion reactions which require cofactor regeneration are already used in industrial chemical production (Kragl *et al.,* 1996). In many cases purified and cross-linked crystalline enzymes are much more tolerant to the varying process conditions than living cells. In addition, the multiple purification steps required by chemical synthesis results in pure fractions of utilisable substrates, which already represents better utilisation of renevable natural resources. However, there are lots of possibilities in the SCREM type processes, which combine thoroughly studied microbial physiology and advanced process control. Transient changes opens an interesting approach to cell regulation and metabolic engineering in different growth conditions. The xylose catabolic pathway in yeasts with different cofactor specificities offers an intruiging research challenge in the future in respect to xylose fermentation.

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