Annals of Microbiology, **57 (1)** 85-92 (2007)

A constitutive catabolite repression mutant of a recombinant *Saccharomyces cerevisiae* strain improves xylose consumption during fermentation

Vasudevan THANVANTHRI GURURAJAN[1], Marie-F. GORWA-GRAUSLUND[2], Bärbel HAHN-HÄGERDAL[2], Isak S. PRETORIUS[1,3]*, Ricardo R. CORDERO OTERO[1**]

[1]Institute for Wine Biotechnology, Stellenbosch University, Private Bag X1, Matieland, Stellenbosch, ZA-7600, South Africa; [2]Department of Applied Microbiology, PO Box 124, Lund University, Lund, SE-221 00, Sweden; [3]The Australian Wine Research Institute, PO Box 197, Glen Osmond, Adelaide, SA 5064, Australia

Received 29 September 2007 / Accepted 20 January 2007

Abstract - Efficient xylose utilisation by microorganisms is of importance to the lignocellulose fermentation industry. The aim of this work was to develop constitutive catabolite repression mutants in a xylose-utilising recombinant *Saccharomyces cerevisiae* strain and evaluate the differences in xylose consumption under fermentation conditions. *S. cerevisiae* YUSM was constitutively catabolite repressed through specific disruptions within the *MIG1* gene. The strains were grown aerobically in synthetic complete medium with xylose as the sole carbon source. Constitutive catabolite repressed strain YCR17 grew four-fold better on xylose in aerobic conditions than the control strain YUSM. Anaerobic batch fermentation in minimal medium with glucose-xylose mixtures and N-limited chemostats with varying sugar concentrations were performed. Sugar utilisation and metabolite production during fermentation were monitored. YCR17 exhibited a faster xylose consumption rate than YUSM under high glucose conditions in nitrogen-limited chemostat cultivations. This study shows that a constitutive catabolite repressed mutant could be used to enhance the xylose consumption rate even in the presence of high glucose in the fermentation medium. This could help in reducing fermentation time and cost in mixed sugar fermentation.

Key words: xylose fermentation, xylitol, *MIG1*, constitutive catabolite repression, *Saccharomyces cerevisiae*.

INTRODUCTION

Xylose is the second major monosaccharide available in nature, constituting about 30% of plant biomass (Hayn *et al*., 1993; Lee, 1997). Many bacteria, yeast and filamentous fungi are capable of xylose utilisation (Chiang and Knight, 1960; Gong *et al*., 1983; Hahn-Hägerdal *et al*., 1993). *Saccharomyces cerevisiae*, the well-characterised industrial fermentation yeast, is unable to utilise xylose. It can, however, grow slowly on xylulose, which is an isomer of xylose, with a growth rate one-tenth lower than on glucose (Chiang *et al*., 1981; Senac and Hahn-Hägerdal, 1990). Availability of a xylose-fermenting *S. cerevisiae* is of importance to the fermentation industry that uses agricultural waste as raw material.

The inability of *S. cerevisiae* to grow on xylose is due to the lack of active enzymes that convert xylose to xylulose. In natural xylose-utilising fungi and yeasts this conversion is brought about by two enzymes – xylose reductase (XR) and xylitol dehydrogenase (XDH) (Chakravorty *et al*., 1962; Bruinenberg *et al*., 1983) – while in bacteria, a single enzyme, namely xylose isomerase, carries out the isomerisation step (Hochster and Watson, 1954). Xylulose is then metabolised through the Pentose Phosphate Pathway (PPP) into either biomass or other fermentation products. When the genes coding for the two enzymes XR and XDH from the natural, xylose-utilising yeast *Pichia stipitis* were introduced into *S. cerevisiae*, growth on xylose was observed, when xylulokinase (XK) was overexpressed. Since then, several attempts have been made to develop a *S. cerevisiae* strain capable of showing as efficient growth and yield on xylose as on glucose (reviewed by Hahn-Hägerdal *et al*., 2001; Jeffries and Jin, 2004).

Xylose, however, being a pentose sugar metabolised through the non-oxidative PPP, which by itself is a low-flux pathway under normal (glucose-grown) conditions (Fiaux *et al*., 2003), limits the efficacy of growth and yield on xylose. Recombinant *S. cerevisiae* strains, in which XR, XDH and XK encoding genes were expressed, have also been reported to be limited by: (*i*) lower affinity for xylose by *S. cerevisiae*'s hexose transporters (Kotyk, 1967; Busturia and Lagunas, 1986; Kötter and Ciriacy, 1993); (*ii*) its low activity pentose phosphate pathway (PPP) (Senac and Hahn-Hägerdal, 1990; Fiaux *et al*., 2003); (*iii*) the inability to oxidize reduced cofactors produced in the initial xylose metabolism (Bruinenberg *et al*., 1983); and (*iv*) reduced energy (ATP) recovery during xylose metabolism (Sonderegger *et al*.,

* Corresponding author. Phone: +61 8 83036610; Fax: +61 8 8303 6601; E-mail address: Sakkie.Pretorius@awri.com.au
**Current address: Department of Biochemistry and Biotechnology, University Rovira i Virgili, Marcel·lí D. Str., Tarragona, E-43007, Spain

2004). In order to improve xylose utilisation by the aforementioned recombinant *S. cerevisiae* strains, genetic and metabolic engineering approaches (Meinander and Hahn-Hägerdal, 1997; Jin *et al*., 2002; Johansson and Hahn-Hägerdal, 2002; Jeppsson *et al*., 2003; Verho *et al*., 2003) have been combined with a mutational approach (Wahlbom *et al*., 2003a) as well as evolutionary engineering (Sonderegger and Sauer, 2003), resulting in mutants that are able to grow on and ferment xylose. Genome-wide transcription analysis of a random mutant (Wahlbom *et al*., 2003b) and an evolutionary mutant (Sonderegger *et al*., 2004) showed the induction of genes involved in transport, redox metabolism, and the lower part of glycolysis.

It has been shown (Meinander and Hahn-Hägerdal, 1997) that xylose utilisation is facilitated by the presence of low amounts of glucose in the medium. Xylose utilisation was impeded either when glucose was depleted or when there was excess. It is difficult to determine the optimal concentration of glucose that can aid efficient xylose utilisation, although a recent report by Öhgren *et al*. (2006) has shown xylose to be co-fermented at glucose levels below 4 g/l in a simultaneous saccharification and fermentation (SSF) process. In general, *S. cerevisiae* prefers glucose to most other sugars in the medium, and metabolises it efficiently. Thus, in the presence of glucose other sugar-metabolising pathways are inhibited, in a process known as carbon catabolite repression or, simply, glucose repression (reviewed by Gancedo, 1998). Therefore, in mixed sugar fermentation involving glucose, the utilisation of other sugars is delayed affecting the efficacy of the process.

We hypothesized that by mimicking a glucose-like environment in xylose-rich conditions, xylose utilisation could be improved. In order to prolong/mimic the glucose-like condition we created constitutive catabolite repression mutants through in-frame deletions within the *MIG1* gene. Östling *et al*. (1996) have shown that deleting one or either of the two regulatory domains in *MIG1* conferred constitutive repression to the strain. In such strain, the glycolytic genes remain induced irrespective of the presence of glucose in the medium. Since xylose shares transporter genes as well as several glycolytic pathway genes with glucose, this condition might prove conducive for improved xylose utilisation. Previously, Roca *et al*. (2004) studied the role of glucose catabolite repression on xylose fermentation through *MIG1* disruption and found a derepressed strain showing improved xylose consumption during mixed sugar fermentation. They also suggested that in a recombinant strain, xylose itself could be a repressive sugar. Belinchon and Gancedo (2003) also reported that xylose causes repression, suggesting that xylose might be recognized as a non-fermentable sugar.

Here we report on the effect of a constitutive glucose-repressed recombinant strain of *S. cerevisiae* on xylose utilisation and fermentation and show that constitutive repression improves xylose consumption even in the presence of high glucose concentration.

TABLE 1 - Yeast strains and plasmids used in this study

Name	Description
Strain	
YUSM	CEN.PK 2-1D *XYL1-2::URA3*
YDM1	YUSM *Δmig1::KanMX4*
YCR5	YUSM *Δmig1* pHMIGΔ5::*HIS3*
YCR17	YUSM *Δmig1* pHMIGΔ17::*HIS3*
YOM	YUSM *Δmig1* pHMIG1::*HIS3*
Plasmid	
pSTAH	YIp5-*Δura3*::*HIS3*:*PGK1*p-*PGK1*t
pHMIG1	YIp5-*Δura3*::*HIS3*:*PGK1*p-*MIG1*-*PGK1*t
pHMIG1Δ5	YIp5-*Δura3*::*HIS3*:*PGK1*p-*MIG1*Δ5-*PGK1*t
pHMIG1Δ17	YIp5-*Δura3*::*HIS3*:*PGK1*p-*MIG1*Δ17-*PGK1*t

MATERIALS AND METHOD

Strains. *Saccharomyces cerevisiae* CEN.PK 2-1D was transformed with *XYL1*, *XYL2* genes from *Pichia stipitis*. This resulted in the strain YUSM 1001d (hereafter referred to as YUSM), which is the control strain used in this study. All strains derived from YUSM for this study are given in Table 1. Plasmids were cloned and amplified in the bacterial host *Escherichia coli* DH5α.

Recombinant DNA methods, plasmid construction and transformation. Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1995). Restriction enzymes (from Roche, Mannheim, Germany or Fermentas, Vilnius, Lithuania), T4 DNA-ligase and Expand Hi-Fidelity DNA polymerase (Roche) were used according to the specifications of the supplier. *Escherichia coli* was transformed as described by Inoue *et al*. (1990), while the lithium acetate method (Gietz *et al*., 1992) was used for yeast transformations.

The *MIG1* gene from the YUSM strain was isolated by the polymerase chain reaction (PCR) technique [using primers MIG1-F (CCCAAGCTTATGCAAAGCCCATATCC) and MIG1-R (GGAAGATCTTCAGTCCAT GTGTGGG)] as a 1533-bp fragment with flanking *Hin*dIII and *Bgl*II sites (underlined above) and cloned into the pGEM-T Easy® vector (Promega, Madison, WI, USA). Removal of a 337-bp fragment between the *Cla*I and *Nhe*I sites resulted in plasmid pGEM-MIG1Δ5. Similarly, removal of a 347-bp fragment between the *Swa*I and *Nhe*I sites resulted in plasmid pGEM-MIG1Δ17 (Östling *et al*., 1996). *Hin*dIII/*Bgl*II digests were used to excise the *MIG1* (1533 bp), *MIG1*Δ5 (1200 bp) and *MIG1*Δ17 (1186 bp) fragments from these respective pGEM constructs. The *MIG1 Hin*dIII/*Bgl*II fragments were blunt-ended by using the Klenow component of polymerase I (Ausubel *et al*., 1995) and inserted into the blunt-ended *Eco*RI site of plasmid pSTAH (Table 1). This resulted in plasmids pHMIG1, pHMIGΔ5 and pHMIGΔ17, respectively, with *MIG1* and its disruptants being under control of the yeast phosphoglycerate kinase I gene (*PGK1*) promoter ($PGK1_p$) and terminator ($PGK1_t$) sequences.

The *MIG1* deletion cassette from the *S. cerevisiae* deletion library strain (BY4742 Δ*mig1*) was obtained using PCR with MIG1-F and MIG1-R primers. This Δ*mig1* cassette was used to delete *MIG1* in YUSM by homologous recombination, thus creating strain YDM1. In order to develop the constitutive catabolite repression strains, *Nsi*I-digested plasmids pHMIG1, pHMIGΔ5 and pHMIGΔ17 were integrated at the *HIS3* locus of YDM1. The resultant strains were named YOM, YCR5 and YCR17, respectively. All gene integrations and deletions were verified by Southern blots (data not shown).

TABLE 2 - Xylose concentrations and metabolite yields measured from anaerobic batch fermentation in mineral medium with 20 g/l glucose and 50 g/l xylose, for the three strains tested (values represented are averages of two individual fermentation)

Strain	Xylose levels (g/l)		Yield from total sugars (g/g)					
	Initial	Consumed	$Y_{S,X}$ *	$Y_{S,Xol}$ †	$Y_{S,Gly}$ ‡	$Y_{S,Ace}$ §	$Y_{S,E}$ **	$Y_{X,Xol}$ ††
YUSM	48.6 ± 0.3	12.6 ± 0.3	0.09 ± 0.003	0.37 ± 0.001	0.06 ± 0.003	0.05 ± 0.005	0.19 ± 0.002	0.95 ± 0.01
YDM1	48.3 ± 0.3	9.2 ± 2.0	0.10 ± 0.012	0.35 ± 0.05	0.07 ± 0.02	0.05 ± 0.002	0.17 ± 0.02	0.99 ± 0.03
YCR17	48.0 ± 0.7	13.5 ± 2.0	0.08 ± 0.00	0.38 ± 0.01	0.05 ± 0.004	0.05 ± 0.001	0.19 ± 0.009	0.96 ± 0.07

* Biomass yield, † xylitol yield, ‡ glycerol yield, § acetate yield, ** ethanol yield, †† xylitol yield from only xylose.
Glucose was totally consumed within 24 h from the start of fermentation for all the three strains. Fermentation was carried out for at least 100 h.

TABLE 3 - Sugar consumption and yield values from N-limited anaerobic chemostat cultivations performed with the three strains (values are averages of duplicate experiments with SD ≤ 10%)

Feed	Strains	FeedSugar (g/l)		Xylose consumed	Yield on total sugars (g/g)						$Y_{X,Xol}$ †
		Glucose	Xylose	(g/l)	Xylitol	Glycerol	Acetate	Biomass	CO_2	Ethanol	
G20X10*	YUSM	20.4	10.5	3.1	0.08	0.01	0.01	0.08	0.45	0.45	0.61
	YDM1	19.8	9.9	2.3	0.09	0.08	0.01	0.09	0.42	0.40	0.80
	YCR17	20.7	9.9	4.3	0.13	0.04	0.01	0.08	0.42	0.41	0.76
G10X10*	YUSM	10.2	9.9	1.7	0.10	0.02	0.01	0.12	0.46	0.44	0.74
	YDM1	9.9	10.2	2.3	0.16	0.04	0.01	0.12	0.43	0.40	0.84
	YCR17	10.5	10.5	2.7	0.16	0.03	0.02	0.17	0.42	0.40	0.79
G2X10*	YUSM	1.8	9.3	0.6	0.11	0.05	0.02	0.16	0.44	0.43	0.48
	YDM1	2.1	9.6	0.9	0.11	0.03	0.04	0.22	0.48	0.39	0.38
	YCR17	2.3	10.2	0.6	0.13	0.04	0.01	0.15	0.53	0.41	0.71

* G20X10 – feed with 20 g/l glucose; G10X10 – feed with 10 g/l glucose; G2X10 – feed with 2 g/l glucose; 10 g/l xylose added to all the three feeds.
† Xylitol yield on xylose only (g/g).

Media and growth conditions. Bacteria were grown in Luria-Bertani (LB) medium (Ausubel *et al*., 1995) at 37 °C. Ampicillin-resistant (Ap^R) *E. coli* transformants were selected on LB medium containing 100 mg/l ampicillin. Yeast strains were cultivated at 30 °C in either a rich medium, YPD (containing in w/v: 1% yeast extract, 2% peptone and 2% glucose), or a synthetic complete medium, SC [containing 2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids (Difco, Detroit, MI, USA), supplemented with essential amino acids (Sherman *et al*., 1983)]. Deletion strains, in which the *MIG1* gene was disrupted, were grown in YPD medium containing 200 mg/l geneticin (G418; Sigma-Aldrich, St. Louis, MO, USA). Solid media contained 20 g/l agar. Yeast strains were stored in rich medium as 15% (v/v) glycerol stocks at –80 °C, while bacterial cultures were stored in LB medium as 40% (v/v) glycerol stocks.

For batch fermentation, a defined mineral medium (Verduyn *et al*., 1992), containing 20 g/l glucose and 50 g/l xylose, was used. Amino acids were added as required for strain auxotrophy (40 mg/l histidine, 40 mg/l tryptophan and 240 mg/l leucine), while ergosterol and Tween 80 were dissolved in boiling 96% (v/v) ethanol to final concentrations of 0.01 and 0.42 g/l, respectively and added to anaerobic cultures. For N-limitation, 5 g/l ammonium sulphate (Verduyn *et al*., 1992) was reduced to 0.6 g/l (Klein *et al*., 1998). For aerobic growth, a single colony was inoculated in appropriate auxotrophic selective media and incubated at 30 °C in a rotary wheel. From exponential-phase cultures, about 10^5 cells/ml were inoculated into 50 ml SC medium and incubated at 30 °C and 200 rpm. Growth was followed as optical density measurements at 600 nm (OD_{600}) at regular intervals, using a UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan).

Fermentation. Inocula for batch fermentation were prepared by inoculating single colonies into a 1-litre baffled shake-flask containing 100 ml defined mineral medium, at 30 °C for 15 h, in an orbital shaker at 150 rpm. Cells were harvested by centrifugation (5000 *x g*, 4 °C, 5 min) and resuspended in sterile water before inoculating into the fermentation medium to an OD_{600} of 0.25. Fermentation was performed in a BIOFLO III (New Brunswick, USA) bioreactor with a working volume of 1 litre, at 30 °C and 200 rpm. Nitrogen gas containing < 5 ppm O_2 (ADR class 2 1A; AGA, Sweden) was passed through the column at a flow rate of 0.2 litre/min, as measured by a gas-flow regulator (Bronkhorst, The Netherlands). The pH of the medium was automatically maintained at 5.5 ± 0.2 by the addition of 3 M potassium hydroxide. Antifoam (Dow Corning, Midland, MI, USA) was added at 0.5% v/v. Batch fermentation was carried out with 20 g/l glucose and 50 g/l xylose in defined mineral medium. Samples taken at regular time intervals

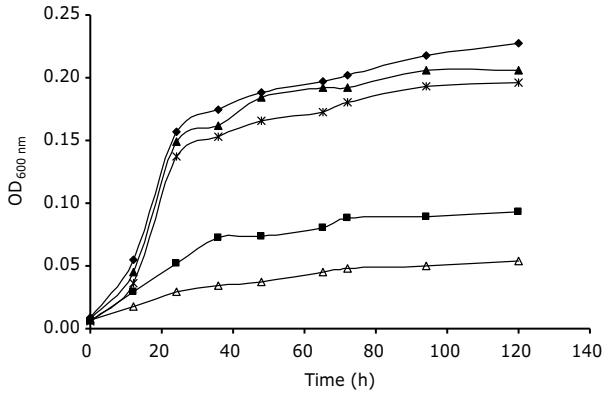

FIG. 1 - Aerobic growth profile of various strains in SC medium with 20 g/l xylose as the sole carbon source, in shake-flask cultures at 30 ºC at 200 rpm. Strains profiled are: YUSM (△), YDM1 (*mig1*) (■), YCR5 (▲), YCR17 (◆), YOM (✳). From these, YUSM, YDM1 and YCR17 were selected for fermentation.

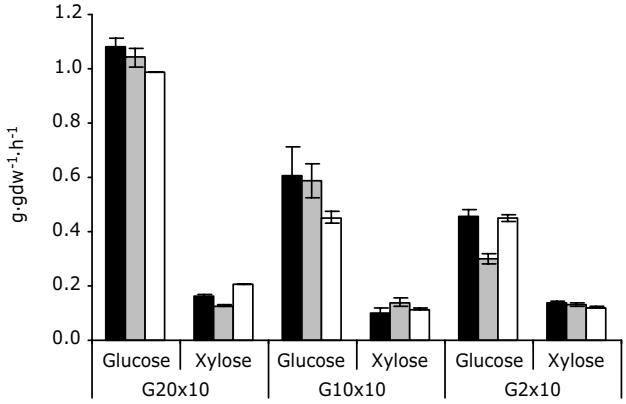

FIG. 2 - Sugar consumption rates of the strains under N-limited chemostat conditions in mineral medium with 10 g/l xylose and 20 g/l glucose (G20X10), 10 g/l glucose (G10X10), and 2 g/l glucose (G2X10). Values, expressed as g/g dry wt/h, are averages of two individual trials (the standard deviations are indicated by error bars). YUSM (black bars), YDM1 (grey bars) and YCR17 (white bars).

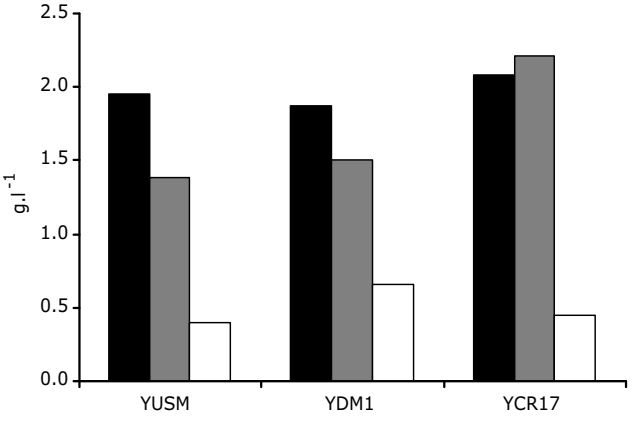

FIG. 3 - Biomass produced during anaerobic N-limited chemostat cultivations with three different feeds – G20X10 (black bars), G10X10 (grey bars), G2X10 (white bars). Values represented are average of duplicate trials (SD ≤ 5%)

were immediately filtered through a 0.22-μm filter (Advantec MFS, Irvine, CA, USA) and stored at −20 °C until further analysis. N-limited chemostat cultivation was started in batch mode, with 20 g/l glucose as the sole carbon source. When glucose was close to depletion, as observed by OD_{600} and CO_2 measurements, fresh N-limited defined mineral medium was fed into the fermenter at a dilution rate (D) of 0.1 h^{-1}. The volume was kept constant at 1 litre by continuous removal of medium from the fermenter using norprene tubing and peristaltic pumps (Masterflex, Cole-Parmer Instruments, Chicago, USA). For each strain three different feed conditions were employed: (*i*) G20X10 – containing 20 g/l glucose and 10 g/l xylose; (*ii*) G10X10 – containing 10 g/l glucose and 10 g/l xylose; and (*iii*) G02X10 – containing 2 g/l glucose and 10 g/l xylose. Steady-state conditions were achieved when the biomass and CO_2 levels remained stable for at least three consecutive volume changes (differing by < 2%). Chemostat cultivation was performed in duplicate for all strains. Samples taken during steady state conditions were immediately filtered through a 0.22-μm filter (Advantec MFS, Irvine, CA, USA) and stored at −20 °C until further analysis.

Analyses of sugars and fermentation products. Substrates consumed and metabolites formed were analysed using a Waters HPLC system (Waters Corporation, Milford, MA, USA) equipped with an Aminex HPX-87H column (Bio-Rad laboratories, Richmond, VA, USA), connected to a refractive index detector (RID-6A, Shimadzu) and an UV detector (Waters-2487; Waters Corporation) in series. A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 45 °C was used to analyse glucose, xylose, xylitol, glycerol, acetate, and ethanol. The measured ethanol values were then corrected by using redox balance equations (Roels, 1983). Off-gas analysis during fermentation was continuously monitored by a CO_2 and O_2 monitor type 1308 (Brüel & Kjær, Nærum, Denmark), using photo acoustic and magneto acoustic detection for CO_2 and O_2 respectively (Christensen *et al*., 1995). Cell dry weight was determined by filtering a known volume of culture broth through a pre-dried (350 W for 4 min in a microwave oven), pre-weighed Supor membrane of 0.45-μm (Gelman Sciences, Ann Arbor, MI, USA). The filter was weighed again after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The difference in the filter weights was used to calculate the dry weight in accordance with the absorbency measured. Dry weights were determined in duplicates. Trehalose and glycogen were assayed as described by Parrou and François (1997), using trehalase and amyloglucosidase (Sigma-Aldrich), respectively.

RESULTS AND DISCUSSION

Development and evaluation of *MIG1* mutants

Improving xylose utilisation is of importance in mixed-sugar fermentation where the presence of easily fermentable sugars could cause catabolite repression. Since glucose (the most common sugar present in fermentation) and xylose share the same transporters in *S. cerevisiae* (Hamacher *et al*., 2002), an attempt was made to mimic and prolong a glucose-like environment in a xylose background. In the YUSM recombinant *S. cerevisiae* strain overexpressing the

P. stipitis xylose reductase and xylitol dehydrogenase genes (Table 1) constitutive catabolite repressed *MIG1* disruptants MIG1△5, MIG1△17 were created according to Östling *et al*. (1996). The strains YCR17 and YCR5, when evaluated for growth in a 20 g/l maltose-containing medium as a means of verifying constitutive catabolite repression, grew comparably slower than YDM1. When the medium contained 10 g/l glucose and 10 g/l maltose, however, YCR17 initially grew more slowly than the other strains but later followed a pattern similar to that of YUSM (data not shown). When the strains were grown in a medium containing 20 g/l xylose as the sole carbon source, YCR17 showed the highest maximum growth rate (0.005 h^{-1}) compared to the control YUSM (0.001 h^{-1}), closely followed by YCR5 and YOM (*PGK1*$_P$-*MIG1*- *PGK1*$_t$) (Fig. 1). YDM1 (growth rate 0.002 h^{-1}) grew two-fold better than the control. Based on growth pattern in xylose medium, three groups of strains emerged – those that were similar to that of (*i*) YCR17, (*ii*) YDM1 and (*iii*) YUSM. Therefore, the xylose fermentation performance of YCR17 and YDM1 compared to YUSM was further investigated under anaerobic batch fermentation.

Previous reports have shown that xylose uptake and utilisation is enhanced by/in the presence of low/optimal amounts of glucose, although the exact amounts are difficult to determine (Kötter and Ciriacy, 1993; Eliasson *et al*., 2000). Meinander and Hahn-Hägerdal (1997) have shown that high concentrations of glucose in the fermentation medium possibly saturate the transport machinery, resulting in very little xylose transport, so that efficient utilisation of xylose occurs only when low glucose levels are present in the medium. Under aerobic conditions a constitutive catabolite repression mutant may maintain the hexose transporters in the active state due to the 'glucose/repression signal', leading to more sugar – in this case, xylose – being transported and consumed by the cell. This was reflected by the fourfold increase in biomass production observed for YCR17 compared to the control strain YUSM.

Batch cultivation

Anaerobic batch cultivation with 20 g/l glucose and 50 g/l xylose, was compared for YUSM, YDM1 and YCR17, to evaluate their xylose fermentation capacities. All three strains fermented glucose within 24 h, while only about one-fifth of the xylose was consumed after 100 h, which could be attributed to the expression of mainly low-affinity transporters during batch cultivations (Roca *et al*., 2004). Xylose is predominantly transported into the cells *via* the high-affinity transporters (Hamacher *et al*., 2002; Gardonyi *et al*., 2003). Roca *et al*. (2004) used a high amount of xylose to overcome the poor affinity and uptake rate of xylose in *S. cerevisiae* (Kötter and Ciriacy, 1993), but xylose consumption by our strains was still comparably low. Xylose consumption was faster in the presence of low glucose in the medium, which is in agreement with the findings of previous studies (Meinander and Hahn-Hägerdal, 1997; Lee *et al*., 2002). Once glucose was depleted, very little xylose was consumed by all three strains. At the end of batch fermentation YCR17 had consumed 8% more xylose (13.5 g/l) than the control strain YUSM (12.6 g/l), and 45% more than the mig1–deleted strain YDM1 (9.2 g/l). Xylose was mainly converted to xylitol, with the xylose to xylitol conversion being more than 90% (Table 2). Glycerol and acetate yields ranged from 0.05 to 0.07 g/g. Ethanol production increased rapidly during the initial stages of fermentation (up to 24 h). Once glucose was consumed, no increase in ethanol production was observed. In terms of biomass yields, the YDM1 strain had a slightly higher yield than YCR17 and YUSM (see Table 2). Although the three strains performed similarly under batch fermentation, there were slight differences in sugar consumption, so that the glucose consumption rate was slower for strain YDM1. But no major differences could be substantiated in batch fermentation.

Nitrogen-limited chemostat cultivation

N-limited chemostat cultivation, in which the growth rate of a culture is limited only by the amount of nitrogen source in the medium, was employed under anaerobic conditions to determine the effect of constitutive catabolite repression on xylose fermentation. Previously Roca *et al*. (2004) had used C-limited chemostat cultivation to study the effect of *mig1* and *mig1mig2* mutants on anaerobic xylose fermentation. In our case, N-limited chemostat cultivation was used as it provided the advantage of possibility to keep the residual concentrations of two sugars at distinct levels (Klein *et al*., 1998), which can help in substantiating the effect, constitutive catabolite repression has, during anaerobic xylose fermentation. The glucose concentration varied from 20 g/l (feed G20X10) and 10 g/l (feed G10X10) to 2 g/l (feed G2X10), with a constant xylose concentration of 10 g/l. The residual sugar concentration in all feed conditions was more than 5 g/l, most of which was xylose. In fact, all three strains consumed glucose to completion under feed condition G2X10. For all strains the rate of glucose consumption decreased with decreasing glucose concentration in the feed (Fig. 2). YCR17 exhibited its highest xylose consumption rate, 0.21 g/g/h, with the G20X10 feed, but no difference with the other two feeds (Fig. 2). Similarly, the control strain displayed its highest xylose consumption rate 0.16 g/g/h in the G20X10 feed. YDM1, consumed xylose with the same rate (0.13-0.14 g/g/h) in all the three feed conditions. The amount of xylose consumed decreased with decreasing amount of glucose in the feed. With feed G20X10, YCR17 consumed more than 40% of the xylose in the medium (Table 3). This was two times more than that consumed by YDM1 and about 10% more than that consumed by YUSM. However, with feed G10X10, the pattern changed; YCR17 still consumed most xylose followed by YDM1 and YUSM, in decreasing order. With feed G2X10, YDM1 was more effective in xylose utilisation even though only 10% xylose was consumed in total. Thus, YCR17 showed increased xylose consumption in presence of high glucose, while xylose consumption was similar to that of the control strain YUSM when the glucose level was lower than the xylose level. Strain YDM1 showed increased xylose consumption when there was less glucose in the medium, as has also been observed by Roca *et al*. (2004).

As was observed in batch fermentation more than 75% of xylose was converted to xylitol (Table 3). Glycerol and acetate yields ranged from 0.01 to 0.08 g/g. Ethanol yields on consumed sugars varied between 0.39 and 0.45 g/g (Table 3). Biomass levels varied with feed conditions (Fig. 3). For the control strain, biomass decreased with decreasing glucose concentration in the feed, a trend followed by the mutant strains. However, YCR17 displayed higher biomass levels when glucose was high/equal in feeds G20X10 and G10X10 than the other two strains. In fact, strain YCR17 produced most biomass when there were equal amounts of glucose and xylose in the feed, which was slight-

ly more than produced by the control strain. At the lowest glucose level, feed G2X10, YDM1 produced most biomass, while that of the other two strains was similar.

Our results emphasise the glucose-dependence of xylose consumption and metabolism under anaerobic conditions (Meinander *et al*., 1999; Eliasson *et al*., 2000). Although xylose transport is less efficient than that of glucose in *S*. *cerevisiae* (Kotyk, 1967; Busturia and Lagunas, 1986), there is evidence that it is not the main limitation for xylose utilisation under anaerobic conditions (Kötter and Ciriacy, 1993). The major problem is rather the metabolic flux through the pentose phosphate pathway (Fiaux *et al*., 2003; Johansson and Hahn-Hägerdal, 2002; Wahlbom *et al*., 2001; Karhumaa et *al*., 2005) as well as the regeneration of reduced co-factors under anaerobic conditions (Bruinenberg *et al*., 1983), which result in xylitol rather than ethanol production. In the YUSM strain background, about 70-90% of xylose consumed in both batch fermentation and chemostat was converted into xylitol. This could also suggest a deficiency in the xylulokinase (XK) activity in this strain (Ho *et al*., 1998; Richard *et al*., 2000; Johansson *et al*., 2001; Toivari *et al*., 2001; Jin *et al*., 2003). The xylulokinase gene *XKS1* was not over-expressed in our strains, and it would be of interest to study the effect of constitutive catabolite repression in such a strain (Eliasson *et al*., 2000; Karhumaa *et al*., 2006).

Biomass production varied between strains and feed conditions in N-limited chemostat. The fact that YCR17 had the highest biomass levels in feeds G20X10 and G10X10 shows that continued repression increases the co-utilisation of glucose and xylose. Nevertheless, the YDM1 strain produced more biomass at low glucose concentration. *MIG1* regulates the high-affinity glucose sensor *SNF3*, which detects low glucose conditions (Kaniak *et al*., 2004). Therefore the increased biomass formation by a *mig1* mutant could suggest the action of *SNF3* at low glucose concentration, which would increase the glucose uptake. Since xylose does not support anaerobic growth (Eliasson *et al*., 2000) there was a significant decrease in biomass production for all three strains with feed G2X10 (low glucose). Energy deficiency and/or ATP depletion has been observed at low dilution rates in N-limited chemostat cultures (Lidèn *et al*., 1995). With low glucose concentration (2 g/l) and xylose being close to a non-fermentable sugar unable to account for anabolic cellular activities, an overall cellular starvation response may result (Thomsson *et al*., 2005). Under such conditions yeast cultures may produce storage carbohydrates such as trehalose and glycogen (Lillie and Pringle, 1980; Panek, 1991; Rose and Vijayalakshmi, 1993). However, neither storage carbohydrate accumulated in the strains investigated (data not shown).

In conclusion the constitutive catabolite repression approach indicated that xylose consumption and metabolism could be considerably increased. This approach will further be evaluated in improved xylose-fermenting strains (Eliasson *et al*., 2000; Wahlbom *et al*., 2003a; Sonderegger *et al*., 2003; Karhumaa *et al*., 2006). Such strains produce more ethanol at a faster rate and a fine-tuned constitutive catabolite repression could lead to faster xylose consumption in the presence of other sugars.

Acknowledgements

This work was funded by South Africa's grapegrowers and winemakers through their investment body, Winetech, as well as by the National Research Foundation of South Africa. VTG was also sponsored by scholarships from the Harry Crossley Foundation (South Africa) and the Swedish Institute (Stockholm, Sweden).

REFERENCES

Ausubel F.M., Brent R., Kingson R.E., Moore D.D., Seidman J.G., Smity J.A., Struhl K. (1995) Current Protocols in Molecular Biology. John Wiley and Sons, New York, USA.

Belinchon M.M., Gancedo J.M. (2003). Xylose and some non-sugar carbon sources cause catabolite repression in *Saccharomyces cerevisiae*. Arch. Microbiol., 180: 293-297.

Bruinenberg P.M., de Bot P.H.M., van Dijken J.P., Scheffers W.A. (1983). The role of redox balances in the anaerobic fermentation of xylose by yeasts. Appl. Microbiol. Biotechnol., 18: 287-292.

Busturia A., Lagunas R. (1986). Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. Gen. Microbiol., 132: 379-385.

Chakravorty M., Viega L.A., Bacila M., Horecker B.L. (1962). Pentose metabolism in Candida *II*. The diphosphoridine nucleotide-specific polyol dehydrogenase of *Candida utilis*. J. Biol. Chem., 237: 1014-1020.

Chiang C., Knight S.G. (1960). Metabolism of D-xylose by moulds. Nature, 168: 79-81.

Chiang L.C., Gong C.S., Chen L.F., Tsao G.T. (1981). D-Xylulose fermentation to ethanol by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 42: 284-289.

Christensen L.H., Schulze U., Nielsen J., Villadsen J. (1995). Acoustic off-gas analyzer for bioreactors: precision, accuracy and dynamics of detection. Chem. Engg. Science, 50: 2601-2610.

Eliasson A., Christensson C., Wahlbom C.F., Hahn-Hägerdal B. (2000). Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL1*, *XYL2*, and *XKS1* in mineral medium chemostat cultures. Appl. Environ. Microbiol., 66: 3381-3386.

Fiaux J., Çakar Z.P., Sonderegger M., Wüthrich K., Szyperski T., Sauer U. (2003). Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Eukaryot. Cell, 2: 170-180.

Gancedo J.M. (1998). Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev., 62: 334-361.

Gardonyi M., Jeppsson M., Liden G., Gorwa-Grauslund M.F., Hahn-Hägerdal B. (2003). Control of xylose consumption by xylose transport in recombinant *Saccharomyces cerevisiae*. Biotechnol. Bioengg., 82: 818-824.

Gietz D., St. Jean A., Woods R.A., Schiestl R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res., 20: 1425.

Gong C.S., Claypool T.A., McCracken L.D., Mauw C.M., Ueng P.P., Tsao G.T. (1983). Conversion of pentoses by yeasts. Biotechnol. Bioengg., 15: 85-102.

Hahn-Hägerdal B., Hallborn J., Jeppsson H., Olsson L., Skoog K., Walfridsson M. (1993). Pentose fermentation to alcohol. In: Saddler, J.N., Ed., Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, pp. 231-290.

Hahn-Hägerdal B., Wahlbom C.F., Gardonyi M., van Zyl W.H., Cordero Otero R.R., Jonsson L.J. (2001). Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilisation. Adv. in Biochem. Engg. Biotechnol., 73: 53-84.

Hamacher T., Johansson B., Gardonyi M., Hahn-Hägerdal B., Boles E. (2002). Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilisation. Microbiology, 148: 2783-2788.

Hayn M., Steiner W., Klinger R., Steinmuller H., Sinner M., Esterbauer H. (1993). Basic research and pilot studies on the enzymatic conversion of lignocellulosics. In: Saddler, J.N., Ed., Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, pp. 33-72.

Ho N.W.Y., Chen Z., Brainard A.P. (1998). Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl. Environ. Microbiol., 64: 1852-1859.

Hochster R.M., Watson R.W. (1954). Enzymatic isomerisation of D-xylose to D-xylulose. Arch. Biochem., 48: 120-129.

Inoue H., Nojima H., Okayama H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. Gene, 96(1): 23-28.

Jeffries T.W., Jin Y.S. (2004). Metabolic engineering for improved fermentation of pentoses by yeasts. Appl. Microbiol. Biotechnol., 63: 495-509.

Jeppsson M., Johansson B., Jensen P.R., Hahn-Hägerdal B., Gorwa-Grauslund M.F. (2003). The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. Yeast, 20: 1263-1272.

Jin Y.S., Jones S., Shi N.Q., Jeffries T.W. (2002). Molecular cloning of *XYL3* (D-xylulokinase) from *Pichia stipitis* and characterization of its physiological function. Appl. Environ. Microbiol., 68: 1232-1239.

Jin Y.S., Ni H., Laplaza J.M., Jeffries T.W. (2003). Optimal growth and ethanol production from xylose by recombinant *Saccharomyces cerevisiae* require moderate D-xylulokinase activity. Appl. Environ. Microbiol., 69: 495-503.

Johansson B., Christensson C., Hobley T., Hahn-Hägerdal B. (2001). Xylulokinase overexpression in two strains of *Saccharomyces cerevisiae* also expressing xylose reductase and xylitol dehydrogenase and its effect on fermentation of xylose and lignocellulosic hydrolysate. Appl. Environ. Microbiol., 67: 4249-4255.

Johansson B., Hahn-Hägerdal B. (2002). The non-oxidative pentose phosphate pathway controls the fermentation rate of xylulose but not of xylose in *Saccharomyces cerevisiae* TMB3001. FEMS Yeast Res., 2(3): 277-282.

Kaniak A., Xue Z., Macool D., Kim J.H., Johnston M. (2004). Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Eukaryot. Cell, 3: 221-231.

Karhumaa K., Hahn-Hägerdal B., Gorwa-Grauslund M.F. (2005). Investigation of limiting metabolic steps in the utilisation of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast, 22: 359-368.

Karhumaa K., Wiedemann B., Hahn-Hägerdal B., Boles E., Gorwa-Grauslund M.F. (2006). Co-utilisation of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microb. Cell Factories, 5:18

Klein C.J.L., Olsson L., Nielsen J. (1998). Nitrogen-limited continuous cultivations as a tool to quantify glucose control in *Saccharomyces cerevisiae*. Enzyme Microb. Technol., 23: 91-100.

Kötter P., Ciriacy M. (1993). Xylose fermentation by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol., 38: 776-783.

Kotyk A. (1967). Properties of the sugar carrier in baker's yeast. 2. Specificity of transport. Folia Microbiol., 12: 121-131.

Lee J. (1997). Biological conversion of lignocellulosic biomass to ethanol. J. Biotechnol., 56: 1-24.

Lee W.J., Kim M.D., Ryu Y.W., Bisson L.F., Seo J.H. (2002). Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol., 60: 186-191.

Lidèn G., Persson A., Gustafsson L., Niklasson C. (1995). Energetics and product formation by *Saccharomyces cerevisiae* grown in anaerobic chemostats under nitrogen limitation. Appl. Microbiol. Biotechnol., 43: 1034-1038.

Lillie S., Pringle J.R. (1980). Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J. Bacteriol., 143: 1384-1394.

Meinander N.Q., Hahn-Hägerdal B. (1997). Influence of cosubstrate concentration on xylose conversion by recombinant, *XYL1*-expressing *Saccharomyces cerevisiae*: a comparison of different sugars and ethanol as cosubstrates. Appl. Environ. Microbiol., 63: 1959-1964.

Meinander N.Q., Boels I., Hahn-Hägerdal B. (1999). Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing *XYL1* and *XYL2* from *Pichia stipitis* with and without overexpression of *TAL1*. Bioresource Technol., 68: 79-87.

Öhgren K., Bengtsson O., Gorwa-Grauslund M.F., Galbe M., Hahn- Hägerdal B., and Zacchi G. (2006). Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. J. Biotechnol., 126: 488-498.

Östling J., Carlberg M., Ronne H. (1996). Functional domains in the Mig1 repressor. Mol. Cell Biol., 16: 753-761.

Parrou J.L., François J. (1997). A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal. Biochem., 248: 186-188.

Panek A.D. (1991). Storage carbohydrates. In: Rose A.H., Harrison J.S. Eds., The Yeasts. Vol. 4, 2nd edn., Academic Press, San Diego. pp. 655-678

Richard P., Toivari M.H., Penttila M. (2000). The role of xylulokinase in *Saccharomyces cerevisiae* xylulose catabolism. FEMS Microbiol. Lett., 190: 39-43.

Roels J.A. (1983). Energetics and kinetics in biotechnology. 1st edn. Elsevier Biomedical Press BV, Amsterdam, The Netherlands

Roca C., Haack M.B., Olsson L. (2004). Engineering of carbon catabolite repression in recombinant xylose fermenting *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol., 63: 578-583.

Rose A.H., Vijayalakshmi G. (1993). Baker's yeast. In: Rose, A.H., Harrison, J.S., Eds., Yeast Technology Vol. 5., Academic Press, San Diego, pp. 357-397.

Senac T., Hahn-Hägerdal B. (1990). Intermediary metabolite concentrations in xylulose- and glucose-fermenting *Saccharomyces cerevisiae* cells. Appl. Environ. Microbiol., 56: 120-126.

Sherman F., Fink G., Hicks J.B. (1983). Methods in yeast genetics. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Sonderegger M., Sauer U. (2003). Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl. Environ. Microbiol., 69: 1990-1998.

Sonderegger M., Jeppsson M., Hahn-Hägerdal B., Sauer U. (2004). Molecular basis for anaerobic growth of *Saccharomyces cerevisiae* on xylose, investigated by global gene expression and metabolic flux analysis. Appl. Environ. Microbiol., 70: 2307-2317.

Thomsson E., Gustafsson L., Larsson C. (2005). Starvation response of *Saccharomyces cerevisiae* grown in anaerobic nitrogen- or carbon-limited chemostat cultures. Appl. Environ. Microbiol., 71: 3007-3013.

Toivari M.H., Aristidou A., Ruohonen L., Penttila M. (2001). Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (*XKS1*) and oxygen availability. Metabol. Engg., 3: 236-249.

Verduyn C., Postma E., Scheffers W.A., van Dijken J.P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast, 8: 501-517.

Verho R., Londesborough J., Penttila M., Richard P. (2003). Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol., 69: 5892-5897.

Wahlbom C.F., Eliasson A., Hahn-Hägerdal B. (2001). Intracellular fluxes in a recombinant xylose-utilising *Saccharomyces cerevisiae* cultivated anaerobically at different dilution rates and feed concentrations. Biotechnol. Bioengg., 72: 289-296.

Wahlbom C.F., van Zyl W.H., Jonsson L.J., Hahn-Hägerdal B., Cordero Otero R.R. (2003a). Generation of the improved recombinant xylose-utilising *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res., 3: 319-326.

Wahlbom C.F., Cordero Otero R.R., van Zyl W.H., Hahn-Hägerdal B., Jonsson L.J. (2003b). Molecular analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilise xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. Appl. Environ. Microbiol., 69: 740-746.