Annals of Microbiology, **57 (4)** 599-607 (2007)

Development and characterisation of a recombinant *Saccharomyces cerevisiae* mutant strain with enhanced xylose fermentation properties

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Received 27 June 2007 / Accepted 26 September 2007

Abstract - The purpose of this study was to help lay the foundation for further development of xylose-fermenting *Saccharomyces cerevisiae* yeast strains through an approach that combined metabolic engineering and random mutagenesis in a recombinant haploid strain that overexpressed only two genes of the xylose pathway. Previously, *S. cerevisiae* strains, overexpressing heterologous genes encoding xylose reductase, xylitol dehydrogenase and the endogenous *XKS1* xylulokinase gene, were randomly mutagenised to develop improved xylose-fermenting strains. In this study, two gene cassettes ($ADH1_P$-*PsXYL1*-$ADH1_T$ and $PGK1_P$-*PsXYL2*-$PGK1_T$) containing the xylose reductase (*PsXYL1*) and xylitol dehydrogenase (*PsXYL2*) genes from the xylose-fermenting yeast, *Pichia stipitis*, were integrated into the genome of a haploid *S. cerevisiae* strain (CEN.PK 2-1D). The resulting recombinant strain (YUSM 1001) overexpressing the *P. stipitis XYL1* and *XYL2* genes (but not the endogenous *XKS1* gene) was subjected to ethyl methane sulfonate (EMS) mutagenesis. The resulting mutants were screened for faster growth rates on an agar medium containing xylose as the sole carbon source. A mutant strain (designated Y-X) that showed 20-fold faster growth in xylose medium in shake-flask cultures was isolated and characterised. In anaerobic batch fermentation, the Y-X mutant strain consumed 2.5-times more xylose than the YUSM 1001 parental strain and also produced more ethanol and glycerol. The xylitol yield from the mutant strain was lower than that from the parental strain, which did not produce glycerol and ethanol from xylose. The mutant also showed a 50% reduction in glucose consumption rate. Transcript levels of *XYL1*, *XYL2* and *XKS1* and the *GPD2* glycerol 3-phosphate dehydrogenase gene from the two strains were compared with real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. The mutant showed 10-40 times higher relative expression of these four genes, which corresponded with either the higher activities of their encoded enzymes or by-product formation during fermentation. Furthermore, no mutations were observed in the mutant's promoter sequences or the open reading frames of some of its key genes involved in carbon catabolite repression, glycerol production and redox balancing. The data suggest that the enhancement of the xylose fermentation properties of the Y-X mutant was made possible by increased expression of the xylose pathway genes, especially the *XKS1* xylulokinase gene.

Key words: mutagenesis, xylose fermentation, xylulokinase, xylitol dehydrogenase, xylose reductase, *XKS1*, *XYL1*, *XYL2*, *Saccharomyces cerevisiae*, yeast.

INTRODUCTION

Efficient conversion of xylose in lignocellulose-based raw materials by recombinant *Saccharomyces cerevisiae* is important for the production of fuel ethanol. It forms an integral part of a world-wide, long-term research effort aimed at the development of an economically viable and sustainable process for the efficient bioconversion of lignocellulose into biofuels, preferably by a single organism in a one-step process known as *consolidated bioprocessing* (Lynd, 1996; Lynd *et al*., 2002).

Naturally occurring *S. cerevisiae* strains cannot ferment xylose. However, they are capable of utilising and fermenting an isomer of xylose, xylulose, at a rate 10-times slower than that of glucose consumption (Wang and Schneider, 1980; Chiang *et al*., 1981; Senac and Hahn-Hägerdal, 1990; Jeppsson *et al*., 1996). In this process, xylulose is metabolised through the pentose phosphate pathway, which feeds into glycolysis and the formation of ethanol. Hence, genes encoding the two-step reduction/oxidation of xylose to xylulose from the xylose-fermenting yeast *Pichia stipitis* have been expressed in *S. cerevisiae* (Kötter and Ciriacy, 1993; Tantirungkij *et al*., 1993). A recombinant *S. cerevisiae* strain, expressing the xylose reductase (XR) gene (*PsXYL1*) and xylitol dehydrogenase (XDH) gene (*PsXYL2*), was able to grow on xylose, albeit very slowly (Kötter *et al*., 1990; Hallborn *et al*., 1991).

The *XYL1* and *XYL2* encoded enzymes have different cofactor preferences. The *P. stipitis* XR enzyme can use both NADPH and NADH, with preference for NADPH

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(Verduyn *et al*., 1985; Rizzi *et al*., 1988; Kostrzynska *et al*., 1998), while its XDH enzyme is exclusively dependent on NAD^+ (Rizzi *et al*., 1989; Kötter *et al*., 1990). Normally, ethanol formation from glucose in *S*. *cerevisiae* is redox neutral, but when transformed with *XYL1* and *XYL2*, the XR and XDH enzymes' differing cofactor specificities led to a lack of NAD^+, especially under anaerobic conditions (Bruinenberg *et al*., 1984). It was found that *S*. *cerevisiae* also requires a carefully up-regulated *XKS1*-encoded xylulokinase activity (XK) to metabololise xylose to ethanol (Ho *et al*., 1998; Eliasson *et al*., 2000; Johansson *et al*., 2001).

In addition to using targeted metabolic engineering approaches (Bailey, 1991) to generate xylose-fermenting strains of *S*. *cerevisiae*, strains overexpressing XR, XDH and XK have been further improved by random evolutionary engineering strategies (Sauer, 2001; Sonderegger and Sauer, 2003; Wahlbom *et al*., 2003a). The potential of evolutionary engineering was recently demonstrated with a xylose-utilising mutant from a non-recombinant *S*. *cerevisiae* strain (Attfield and Bell, 2006). This strain had increased xylose reductase and xylitol dehydrogenase activities with no apparent increase in xylulokinase activity, although the anaerobic xylose fermentation properties of this strain have not been reported.

In light of the results reported to date regarding the development of xylose-fermenting *S*. *cerevisiae* strains, we believe that it is reasonable to explore ways of further improvement of these strains by subjecting them to a combination of genetic engineering, metabolic engineering, mutagenesis and/or directed evolutionary engineering strategies. The purpose of the present study was to overexpress the *P*. *stipitis XYL1* xylose reductase gene and *XYL2* xylitol dehydrogenase gene in a haploid laboratory strain of *S*. *cerevisiae* (CEN.PK 2-1D) without overexpressing a cloned version of the endogenous *S*. *cerevisiae XKS1* xylulokinase gene, and then to mutate the resulting recombinant strain. Therefore, there are two fundamental aspects that set this study apart from previously published or patented work on the development of xylose-fermenting *S*. *cerevisiae* strains. First, the rationale for conducting this investigation in a *haploid* laboratory strain as opposed to a more robust industrial strain with greater commercial application was to provide more scientific flexibility and opportunity for in-depth genetic analyses and research, which could later be translated and extended to industrial strains. Second, the reason why a cloned version of the endogenous *XKS1* gene was not overexpressed together with the heterologous *XYL1* and *XYL2* genes was to demonstrate that it is possible to generate a xylose-utilising strain by combining a metabolic engineering approach with classical random mutagenesis. Following this strategy, we succeeded in developing a recombinant *S*. *cerevisiae* mutant strain with a 20-fold increase in aerobic growth on xylose. The strain fermented xylose into ethanol and glycerol, rather than xylitol.

MATERIALS AND METHODS

Recombinant DNA methods, transformation. Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1995). Restriction enzymes (Roche, Mannheim, Germany), T4 DNA-ligase (Promega, Madison, WI, USA) and Takara *Ex-Taq* DNA polymerase (TakaRa Bio, Shiga, Japan) were used according to the specifications of the supplier. Bacteria were transformed as described by Ausubel *et al*. (1995), while the lithium acetate method (Gietz *et al*., 1992) was used for yeast transformations.

Microbial strains, plasmids and media. The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1. *Escherichia coli* DH5α was used for the amplification of the yeast integrating plasmid pUSM 1001 (Thanvanthri Gururajan *et al*., 2007). This plasmid carries two gene cassettes, containing the *P*. *stipitis* xylose reductase (*$ADH1_P$-PsXYL1-$ADH1_T$*) and xylitol dehydrogenase (*$PGK1_P$-PsXYL2-$PGK1_T$*) genes. The *XYL1* and *XYL2* genes are placed under the control of the *S*. *cerevisiae* alcohol dehydrogenase I (*ADH1*) and phosphoglycerate kinase I (*PGK1*) regulatory sequences, respectively. Plasmid pUSM 1001 was stably integrated into the genome of the haploid *S*. *cerevisiae* strain CEN.PK 2-1D to generate recombinant strain YUSM 1001 (hereafter referred to as YUSM). Mutant strain Y-X was derived from the recombinant YUSM strain after it was mutagenised by ethyl methane sulfonate (EMS).

Yeast strains were grown in rich YP (10 g/l yeast extract, 20 g/l peptone) media containing either 20 g/l glucose (YPD), xylose (YPX), sucrose (YPS), raffinose (YPR), maltose (YPM) or glycerol (YPG), and stored in glycerol stocks at -80 °C. Yeast transformants were grown in a synthetic complete (SC) medium [6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, MI, USA), 20 g/l glucose (SCD) or 20 g/l xylose (SCX)] supplemented with amino acids essential for selection (50 mg/l tryptophan, 240 mg/l leucine, 50 mg/l histidine, and/or 40 mg/l uracil). Yeast strains were also grown in defined mineral medium (Verduyn *et al*., 1992). White, ampicillin-resistant (Ap^R) *E*. *coli* transformants were grown and selected in Luria-Bertani (LB) medium containing 100 mg/l ampicillin and 30 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Ausubel *et al*., 1995). Solid media contained 20 g/l agar (Difco). Yeast cultures were grown at 30 °C and bacteria at 37 °C.

Development and characterisation of mutant Y-X. Recombinant *S*. *cerevisiae* strain YUSM was subjected to mutagenesis with ethyl methane sulfonate (EMS; Sigma-Aldrich, St. Louis, MO, USA). Yeast cells were incubated with EMS for 80, 100, 120 and 140 min to obtain survival rates of between 10 and 60%. Samples withdrawn at these time points were pooled together and plated on YPX plates at a concentration of 10^6 cells per plate. The plates were incubated at 30 °C for 5 days. At the end of incubation, xylose-utilising colonies appeared at a rate of 1-5/10^8 cells. The colonies were replicaplated onto YPX plates and incubated at 30 °C for 2 days, at the end of which the fastest growing mutant culture was isolated and named *S*. *cerevisiae* Y-X.

To characterise mutant Y-X, it was crossed with the control strains CEN.PK 113-7A and YUSM 1001d (Table 1) by a replicaplating method (Sherman *et al*., 1991) on selective SCD medium. The diploid strains obtained were then plated onto rich agar medium with different carbon sources, viz. glucose (YPD), xylose (YPX), sucrose (YPS), raffinose (YPR), maltose (YPM) and glycerol (YPG) and incubated at 30 °C under aerobic and anaerobic conditions.

TABLE 1 - Yeast strains and plasmids used in this study

Strains	Description	Source/Reference
CEN.PK 2-1D	*MATα*; *ura3*-52; *trp1*-289; *leu2*-3_112; *his3*△1; *MAL2-8*^{C}; *SUC2*	Entian and Kötter (1998)
CEN.PK 113-7A	*MATa; URA3*; *LEU2*;*TRP1*; *his3*△1; *MAL2-8*^{C}; *SUC2*	IWBT, Stellenbosch
YUSM	CEN.PK 2-1D: pUSM 1001	Thanvanthri Gururajan *et al*. (2007)
YUSM 1001d	*MATa; URA3*; *LEU2*;*TRP1*; *his3*△1; *MAL2-8*^{C}; *SUC2*: pUSM 1001	This work
CENPK 113-7A/Y-X	Diploid strain created by mating CEN.PK 113-7A with mutant Y-X	This work
CENPK 2-1D/YUSMd	Diploid strain created by mating CEN.PK 2-1D and YUSM 1001d	This work
YUSM d/Y-X	Diploid strain created by mating YUSM 1001d with mutant Y-X	This work
pUSM 1001	YIp5; *$ADH1_P$-PsXYL1-$ADH1_T$; $PGK1_P$-PsXYL2-$PGK1_T$*	Thanvanthri Gururajan *et al*. (2007)

For anaerobic conditions, anaerobic jars were used together with the AnaeroGen™ anaerobic system (Oxoid, Hampshire, England). After 40 h incubation for aerobic plates and 72 h for anaerobic plates, the growth of the cultures was evaluated visually. The fastest growing mutant, Y-X, was selected for further study.

Growth and fermentation. To compare the aerobic growth profile of YUSM and Y-X, the cultures were initially grown until mid-exponential phase in tubes containing SCD medium at 30 °C in a rotary wheel. The cells were then inoculated into Erlenmeyer flasks (250 ml) containing 50 ml of defined mineral medium (Verduyn *et al*., 1992), with 50 g/l xylose as the sole carbon source, to an initial optical density (measured at 600 nm; OD_{600}) of 0.01. Each culture was grown in triplicate. The flasks were incubated at 30 °C and 200 rpm in an orbital shaker. Growth was tracked by sampling at regular intervals and measuring the OD_{600} values using a UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan). For anaerobic fermentation, cells of Y-X and YUSM were pre-grown until late exponential phase in defined mineral medium with 20 g/l glucose in shake flasks at 30 °C and 200 rpm. The cells were then collected by centrifugation (3000 *x g*, 4 °C), washed with sterile water and inoculated at a concentration of 0.05 g/l into the bioreactor. Fermentation was carried out in a BioFlo III fermentor (New Brunswick Scientific, New Brunswick, USA) containing defined mineral medium with 20 g/l glucose and 50 g/l xylose in a total working volume of 1 litre. The medium was supplemented with essential amino acids (according to the auxotrophic requirements of the strains), and ergosterol and Tween 80 for anaerobic growth. Antifoam (Dow Corning, Midland, MI, USA) was added at a concentration of 0.5 ml/l. Fermentation was carried out at 30 °C and 200 rpm, with a pH of 5.5 ± 0.2 maintained by automatic addition of 3 M potassium hydroxide. Anaerobic conditions were maintained by the continuous sparging of nitrogen gas (less than 5 ppm oxygen, ADR class 2 1A; AGA, Malmö, Sweden) at a flow rate of 0.2 ml/min, controlled by a mass flow meter (Bronkhorst High-Tech, Ruurlo, The Netherlands). Carbon dioxide and oxygen levels in the bioreactor were analysed by an acoustic off-gas analyser (Type 1308, Brüel & Kjær, Nærum, Denmark) (Christensen *et al*., 1995). Samples were withdrawn at regular intervals and filtered through a 0.22 µm filter (Advantec MFS, CA, USA) before being stored at -20 °C until further analysis.

Chemical and sequence analyses. Acetate, ethanol, glucose, glycerol, xylitol and xylose were analysed using high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA) with an Aminex P87-H ion-exchange column (Bio-Rad laboratories, Hercules, CA, USA) connected in series to a refractive index detector (RID-6A, Shimadzu, Kyoto, Japan). The column temperature was 45 °C, while the mobile phase was 5 mM sulphuric acid at a flow rate of 0.6 ml/min. Ethanol concentrations were corrected for evaporation by using the degree of reduction balance. No other products were detected by HPLC. 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. Dry weights were determined in duplicates.

For analysis of gene sequences, primers were designed to amplify both the promoter region and the open reading frame (ORF) of the gene by the polymerase chain reaction (PCR) technique. The PCR products were cloned into the sequencing vector pGEM-T Easy® (Promega) and transformed into *E. coli* DH5α. Transformants were grown on LB agar plates containing ampicillin and X-gal for selection of white (indicating the presence of an inserted DNA fragment in the pGEM-T Easy vector) Ap^R colonies. Plasmids were purified with Qiaprep® Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) and sequenced. Cycle sequencing was performed using the BigDye‘ Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and reactions were analysed in either an ABI3100 (Applied Biosystems) automated sequencer or in a Genetic analysis system SCE2410 (SpectruMedix LLC, State College, PA, USA).

Enzyme assay. Crude cell extracts were prepared from fermenting cells (Eliasson *et al*., 2000). Protein concentrations were determined by using the BCA Protein Assay Kit® (Pierce, Rockford, IL, USA) with bicinchoninic acid (BCA) as standard. Xylose reductase and xylitol dehydrogenase activities were determined by adapting previously described assay procedures (Eliasson *et al*., 2000) for use in a microtitre plate reader (Powerwave-X, Bio-tek Instruments, Winooski, VT, USA) whereby the assay mix had a final volume of 0.2 ml. Assays were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme needed to either reduce or oxidise 1 µmol of NAD(P)H per min under the specified conditions.

RNA extraction and real time analysis. Total RNA was obtained from selected fermentation samples of YUSM and Y-X by using the FastRNA® Pro Red Kit and FastPrep® Instrument (Qbiogene, Morgan Irvine, CA, USA), according to the manufacturer's protocol. RNA concentrations and purity were determined by using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and by agarose gel electrophoresis. To eliminate contaminating DNA in samples, DNAaseI (Roche) treatment was performed when necessary. Complementary DNA (cDNA) from samples of total RNA was synthesised by using the reverse transcriptase from Superscript III RT-PCR kit (Invitrogen, Carlsbad, CA, USA), following the instructions in the manual. cDNA concentrations were measured using the NanoDrop® ND-1000 spectrophotometer and ca. 70 ng of cDNA was used as a template for real-time PCR experiments, carried out in a LightCycler® (Roche) instrument. Transcripts of *XYL1*, *XYL2*, *XKS1* and *GPD2* (encoding glycerol 3-phosphate dehydrogenase) were analysed from samples obtained during fermentation. *ACT1*, encoding β-actin, was used as the housekeeping gene to normalise expression values (Giuletti *et al*., 2001; Bleve *et al*., 2003; Divol *et al*., 2006). Primers used for the synthesis of probes for each of these genes were designed using the software available at the primer3© website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky, 2000) and are listed in Table 2. The *ACT1* primers used were the same as those reported by Divol *et al*. (2006). Reactions were carried out in capillaries containing the template cDNA, primers and SYBR-green mix from Quantitect® SYBR-green PCR mix (Qiagen), according to manufacturer's instructions. The reaction steps included the following: initial denaturation at 95 °C for 15 min, followed by 50 cycles at each of the following temperatures and times – 95 °C for 15 s; 56 °C for 10 s; 72 °C for 10 s. After the completion of the PCR reaction, melting curve analysis was performed. Samples were heated to 95 °C, cooled to 56 °C for 15 s before being ramped back to 95 °C in 0.1 °C/s increments. The melting temperatures of the samples (T_m) were determined using the Lightcycler Data Analysis® software. No primer-dimers were observed. Data obtained from the real-time PCR runs were analysed using the data analysis software provided by the manufacturer. The relative expression levels of the genes of interest in YUSM and Y-X were determined using the mathematical model proposed by Pfaffl (2001) and the assumption-free method proposed by Ramakers *et al*. (2003).

TABLE 2 - Primers used for synthesis of probes for real-time PCR analysis

Name	Sequence
*Ps*XYL1-F646(Q)	5′ – TTC GGT CCT CAA TCT TTC GT – 3′
*Ps*XYL1-R758(Q)	5′ – GCT GGA GAC TTA CCG TGC TT – 3′
*Ps*XYL2-F441(Q)	5′ – AGA CCA CGT CAG CTT GGA AC – 3′
*Ps*XYL2-R567(Q)	5′ – AAG ACC AAC AGG ACC AGC AC – 3′
XKS1-F1395(Q)	5′ – TTC AAA CGC AAG CTC ACA AC – 3′
XKS1-R1528(Q)	5′ – CGT TTT TAG AAG CCC CAC CT – 3′
GPD2-F951(Q)	5′ – TCA AAG GCT GGG TTT AGG TG – 3′
GPD2-R1123(Q)	5′ – AGG CTG ACT TAC CGG TCT TG – 3′

RESULTS

Development of a xylose-fermenting recombinant *Saccharomyces cerevisiae* mutant

Saccharomyces cerevisiae strain type CEN.PK 2-1D was transformed with the integrative plasmid pUSM1001, which contained the *P. stipitis XYL1* and *XYL2* genes, to create the reference strain YUSM. This transformant contained a single copy of the endogenous *XKS1* gene. For developing the mutant, EMS was used as the mutagen with growth on xylose plates as the screening procedure. After 5 days of incubation at 30 °C, colonies appeared at a rate of 1-5/10^8 cells. From a subsequent second screening on xylose medium, the fastest growing strain was isolated and named Y-X.

Growth analysis

Growth of strains Y-X and YUSM was assessed in defined mineral medium with xylose (20 g/l) as the sole carbon source (Fig. 1) at 30 °C and 200 rpm. The mutant strain Y-X had a growth rate of 0.042/h on xylose, which represents a more than 20-fold increase compared to the parental strain YUSM.

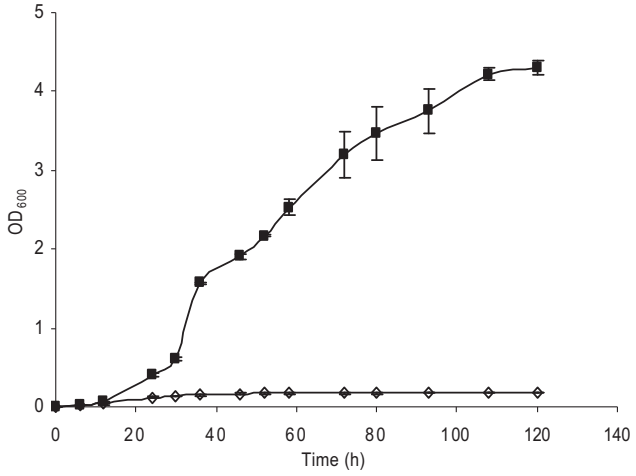

FIG. 1 - Growth profile of the two recombinant *Saccharomyces cerevisiae* strains - the parental strain, YUSM (◇), and the mutant strain, Y-X (■) - in SCX medium at 30 °C and 200 rpm in orbital shaker. Values represented are average of triplicates and the standard deviation (SD) is indicated by error bars.

Genetic analysis

The mutated strain was crossed with mating strains CEN.PK 113-7A and YUSM 1001d to obtain a heterozygous diploid CEN.PK113-7A/Y-X and a homozygous diploid YUSMd/Y-X, respectively. To evaluate the dominant/recessive characteristics of the xylose phenotype, cell growth was observed in rich media with different carbon sources, as shown in Fig. 2. In all cases, growth differences were visually observed for the homozygous diploid and the heterozygous diploid. Similar results were obtained when diploids isolated independently from identical crosses were tested. This suggests that the xylose phenotype was dominant. Growth of the various strains on solid media also indicated that the mutant (and its derivative diploids) did not show any difference in utilising carbon sources other than xylose.

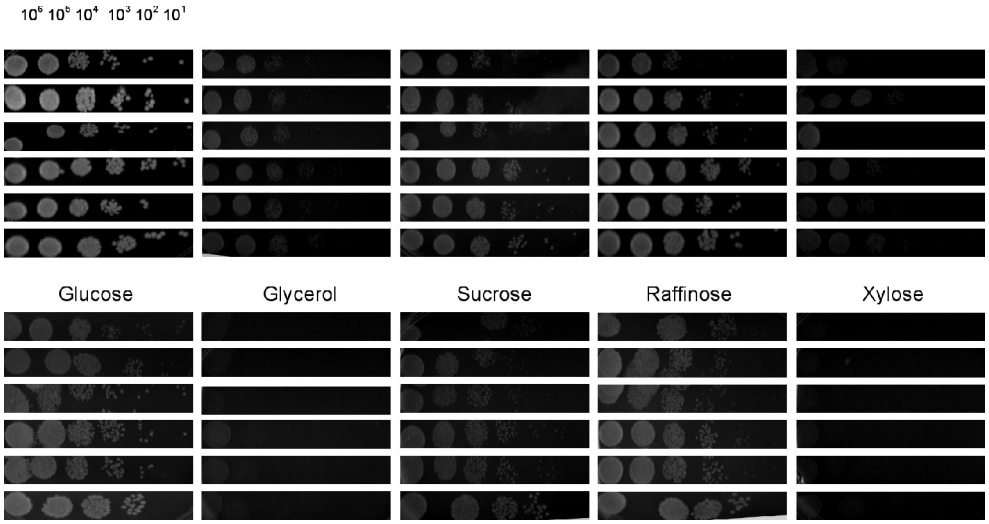

FIG. 2 - Growth profile of the different haploid and diploid *Saccharomyces cerevisiae* strains developed, on rich solid media with different carbon sources. The top six rows represent strains grown aerobically, while the bottom six represent anaerobic growth. In both sets, the strains represented are: (from top to bottom) YUSM, Y-X, CEN.PK, CEN.PK/Y-X, CEN.PK/YUSM, YUSM/Y-X respectively. Each strain was serially diluted from 10^6 cells and spotted sequentially from left to right. Aerobic growth observed after 48 h and anaerobic growth observed after 72 h.

Fermentation

Anaerobic batch cultivation with the X-Y mutant and its parental strain YUSM was performed in controlled bioreactors using defined mineral medium containing 20 g/l glucose and 50 g/l xylose, supplemented with ergosterol and Tween 80 necessary for anaerobic growth of *S. cerevisiae* and the requisite amino acids as specified in the *Materials and Methods* section. The X-Y mutant consumed xylose twice as fast as its parental strain (Fig. 3). On the other hand complete glucose consumption took twice as long for the mutant strain as for the parent strain. Once glucose was depleted xylose consumption became negligible for the parental strain YUSM. It converted xylose mainly to xylitol, while ethanol was primarily formed from glucose (Figs. 3 and 4). Glycerol formation was in the range of 2 g/l. On the contrary, the mutant strain Y-X continued xylose consumption even after glucose depletion. It utilised more xylose and converted the sugar to ethanol and glycerol at the expense of xylitol formation (Fig. 4). While YUSM had a xylitol yield of 0.37 g/g sugar consumed, the corresponding figure for Y-X was only 0.20 g/g consumed sugar. For both the strains, acetate and biomass production were negligible under the chosen conditions.

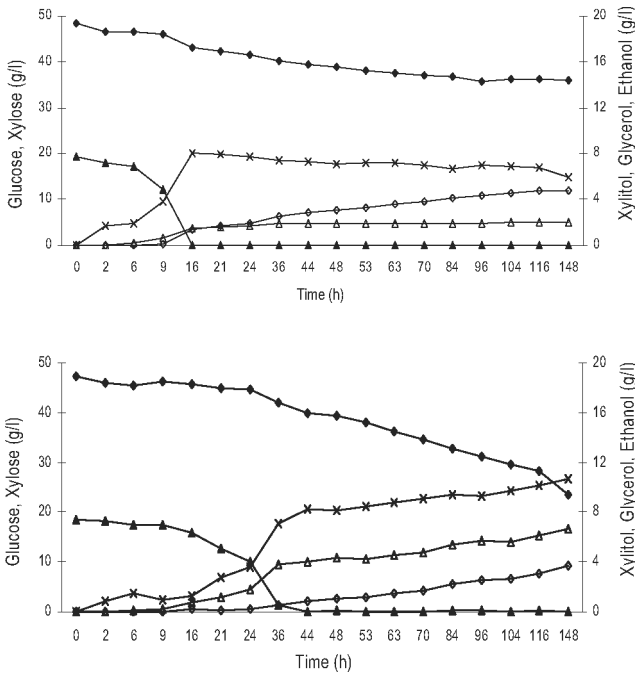

FIG. 3 - Sugars utilised and metabolites formed during batch fermentation in minimal medium with 20 g/l glucose and 50 g/l xylose under complete anaerobic conditions at 30 °C and 200 rpm with parent strain YUSM (top chart) and mutant strain Y-X (bottom chart). Glucose -▲; Xylose -◆; Xylitol -◇; Glycerol -△; ethanol –x. Values represented are averages of duplicate trials (SD < 5%).

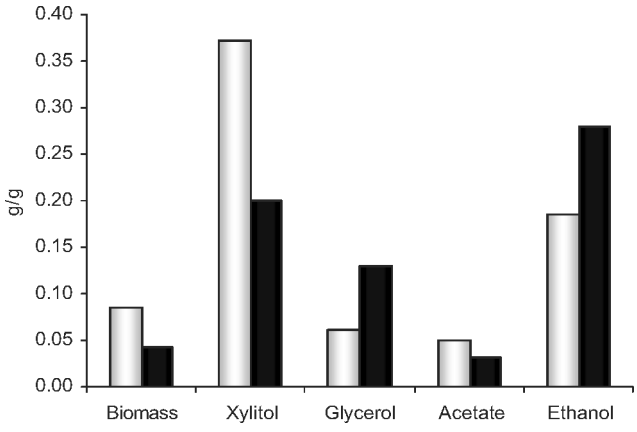

FIG. 4 - Yields of various metabolites formed from total sugars consumed, expressed in g/g, during anaerobic fermentation by YUSM (white bars) and Y-X (black bars). Values represented are averages of two individual experiments (SD < 5%).

TABLE 3 - Specific enzyme activities of the control strain and mutant during fermentation with glucose and xylose at 30°C

Strains	XR		XDH
	NADPH	NADH	
YUSM	0.15 ± 0.01	0.056 ± 0.01	0.97 ± 0.002
Y-X	0.32 ± 0.02	0.167 ± 0.02	4.35 ± 0.01

Enzyme activity

The XR and XDH activities for the two strains are summarised in Table 3. The XR activity was determined with the two cofactors, NADPH and NADH. Both NADPH- and NADH-dependent XR activities for Y-X were at least twice as high as those for YUSM. Furthermore, the XDH activity for Y-X was 5-fold increased. The XR/XDH ratio for Y-X was between 0.04 (NADH) and 0.07(NADPH), while for YUSM it was between 0.06 (NADH) and 0.16 (NADPH).

Transcriptional expression of selected genes

Because the mutant showed increased xylose utilisation and glycerol production, the transcriptional expression of genes in the xylose metabolic pathway – *XYL1, XYL2* and *XKS1* – and the gene that is functional during anaerobic glycerol formation – *GPD2* – (Ansell *et al*., 1997; Björkqvist *et al*., 1997; Valadi *et al*., 2004) were analysed by real-time RT-PCR. Selected samples from anaerobic fermentation representing the glucose phase (when both glucose and xylose were present in the bioreactor), and the xylose phase (when glucose was completely absent) were used to isolate total RNA. Complimentary DNA (cDNA) was reverse-transcribed from these samples. Real-time RT-PCR was performed for each of the four genes mentioned above, using gene-specific primers. The PCR efficiencies were usually ≥80%, determined by using the LinRegPCR© program (Ramakers *et al*., 2003) in order to follow an assumption-free method of calculating the relative expression. Based on the calculations, the relative expression profiles of the four genes of the X-Y mutant to those of the parent strain YUSM are presented in Fig. 5. All four genes tested showed higher levels of expression in Y-X at all four time points. The increased levels of *XYL1* remained consistent throughout the fermentation, while *XYL2* transcript levels fluctuated between time points. *XKS1* showed the highest increase in expression followed by *GPD2* at 96 h. *XKS1* levels were markedly higher when glucose was completely depleted.

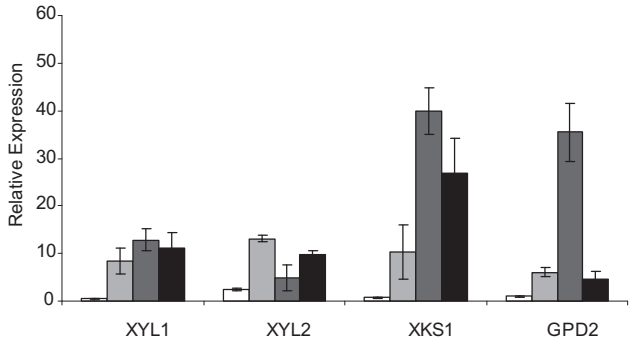

FIG. 5 - Relative expression levels of four genes (given in the X-axis) of Y-X to that of YUSM during fermentation in minimal medium with 20 g/l glucose and 50 g/l xylose under anaerobic conditions, as determined by real-time PCR analysis of cDNA transcripts obtained during 2 h (white bars), 24 h (dotted bars), 96 h (vertical striped bars) and 144 h (diagonal striped bars) of fermentation. SD represented as error bars.

DISCUSSION

Most studies aimed at the development of xylose-fermenting *S. cerevisiae* strains report on strains overexpressing the first three genes involved in xylose conversion, *viz*. *XYL1, XYL2* and *XKS1*. Among the various strategies established for engineering yeast strains, random mutagenesis is an approach wherein the desired characteristic could be obtained without the knowledge of the exact factor(s) responsible for that desired change. Previously, Wahlbom *et al*. (2003a) randomly mutagenised a recombinant *S. cerevisiae* strain overexpressing all three genes of the xylose pathway and developed a mutant with improved properties. The current study was performed to create a random mutant of a haploid recombinant *S. cerevisiae* strain overexpressing the *P. stipitis XYL1* and *XYL2* genes without overexpressing a cloned version of the endogenous *XKS1* gene and to evaluate its performance during anaerobic xylose fermentation. This could be seen in light of the recent development of a non-recombinant *S. cerevisiae* strain that showed increased XR and XDH (but not XK) activities and improved xylose growth by using a directed evolutionary engineering strategy (Attfield and Bell, 2006). Thus the parent strain YUSM was randomly mutagenised by EMS treatment. Since faster xylose utilisation was one of the desired goals, screening the mutants for growth on xylose plates constituted an effective selection procedure. The mutant strain, Y-X, showed a 20-fold increase in growth rate under aerobic conditions and more than twice the amount of xylose consumption during anaerobic fermentation. The mutant strain also showed slower glucose utilisation than the parent during fermentation, similar to that reported by Sonderegger *et al*. (2004). However, growth profiles of the mutant strain Y-X did not differ on other carbon sources tested.

During anaerobic batch fermentation, the Y-X mutant was able to assimilate more xylose than the parent. This could be due to increased XR and XDH activities as reported by Eliasson *et al*. (2001), Jeppsson *et al*. (2003) and Karhumaa *et al*. (2007). When the enzyme activities for the mutant and the parent strains were compared, it was found that the mutant had at least 2-times more XR activity and 4-times more XDH activity than the parent. Real-time RT-

PCR analysis showed a 10-fold increase of *XYL1* and *XYL2* expression for Y-X when compared to those of YUSM. The ratio of XR/XDH in the Y-X mutant was closer to 0.06 as described by Walfridsson *et al*. (1997) for efficient anaerobic xylose fermentation to ethanol. The reduction in xylitol levels, coupled with an increase in ethanol and glycerol suggested an altered cofactor affinity for the XR reaction, probably an increased preference for NADH. However, when XR activity was measured with the two cofactors, the increase was found to be proportional for both, meaning that the enzyme still preferred NADPH. Previously, Kuyper *et al*. (2004) proposed that at a NADPH:NADH ratio of one, the by-products for anaerobic xylose fermentation would be xylitol, glycerol and ethanol. However, Y-X showed 2-times more NADPH-dependent activity than NADH-dependent activity (Table 3). Sequence verification of the two ORFs and the respective promoter regions from the Y-X mutant did not show any mutation(s).

The altered by-product profile for the Y-X mutant suggested a shift in the metabolic flux towards redox balancing, as more glycerol was formed. For YUSM, which lacked XK overexpression, xylitol was the major by-product. It fits with the well-documented importance of xylulokinase overexpression for xylose fermentation to products other than xylitol (Ho *et al*., 1998; Eliasson *et al*., 2000; Richard *et al*., 2000; Johansson *et al*., 2001; Toivari *et al*., 2001; Jin *et al*., 2003; Karhumaa *et al*., 2005). Real-time RT-PCR analysis revealed a 40-fold increase in *XKS1* transcripts for Y-X, but subsequent sequence verification of the ORF together with the promoter region did not show any mutations. Thus, the increase in *XKS1* transcript levels can probably be ascribed to mutations in other regulatory genes or an increase in copy number. Increased glycerol formation by Y-X suggested an increase in the glycerol flux and since *GPD2* is the gene involved in glycerol production under anaerobic conditions (Ansell *et al*., 1997; Björkqvist *et al*., 1997; Valadi *et al*., 2004), transcripts of this gene were compared between YUSM and Y-X. The results showed *GPD2* levels to be at least 5-times higher in Y-X (Fig. 5). Glycerol formation could also be due to redox-related changes during fermentation. A number of genes responsible for redox maintenance are present in the yeast (Bakker *et al*., 2001; Rigoulet *et al*., 2004). However, a few have been considered to be of importance during xylose fermentation. The coding sequences and promoters of various redox-related genes, mainly the endogenous aldose reductases (Träff *et al*., 2002; Träff-Bjerre *et al*., 2004) were verified but no mutation(s) could be determined.

Although the presence of endogenous xylose pathway genes (Toivari *et al*., 2004 and references therein) had been established in *S*. *cerevisiae*, most studies reporting on improving xylose utilisation and fermentation by *S*. *cerevisiae* focussed on the expression of heterologous genes and the development of recombinant yeasts. Recently Attfield and Bell (2006) reported a non-recombinant *S*. *cerevisiae* strain evolutionarily engineered to utilise xylose. Compared with the parent strain this strain exhibited a significant increase in growth, which was coupled with a 4-fold and 80-fold increase in XR and XDH activities, respectively. However, there were no apparent differences in XK activity between the parent and the evolved strains. No data of xylose fermentation for the non-recombinant yeast has been reported so far. The parent strain used in the current study is similar to the aforementioned strain in that the XK was not overexpressed. However, due to a combination of metabolic engineering and random mutagenesis, the mutant strain Y-X displayed increased xylose utilisation and improved anaerobic xylose fermentation properties. Under anaerobic xylose fermentation conditions, the mutant showed increased levels of XR, XDH and XK. The development of this mutant has reiterated the requirement of increased xylulokinase activity for anaerobic xylose fermentation to obtain products other than xylitol. Based on the physiological characteristics exhibited by Y-X, attempts were made to establish the mutation(s) in Y-X, in vain. However, genome-wide characterisation analysis of previous random mutants (Wahlbom *et al*., 2003b) and evolutionary mutants (Sonderegger *et al*., 2004) have shown that the changes were not specific to a specific gene/pathway but were widespread at different metabolic and regulatory levels resulting in the improved characteristic. As none of the structural genes investigated showed any mutations, it could be suggested that there might have been an increase in copy numbers. Proteome analysis might reveal the presence, if any, of post-translational modifications in the mutant; but the work previously reported (Salusjärvi *et al*., 2003) has shown that the changes were widespread at the protein level too.

In conclusion, for the past decade or so, it was widely believed that the only way to construct a xylose-fermenting *S. cerevisiae* strain was to over-express the endogenous *XKS1* xylulokinase gene together with heterologous genes encoding xylose-reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*). Therefore, the present report disproved this dogma. First, by combining genetic engineering and random mutagenesis, we demonstrated in this paper the efficacy of an integrative approach to strain development. Second, by mutating a *XYL1-XYL2* recombinant *S. cerevisiae* strain without the overexpression of a cloned copy of *XKS1*, we showed that there is a novel way of circumventing certain restrictions that might be associated with patented *XYL1-XYL2-XKS1* overexpression strains and approaches. We believe that our method to mutate a recombinant strain represents a novel approach to the development of *S. cerevisiae* strains with superior xylose-fermentation capabilities.

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 sponsored by scholarships from the Harry Crossley Foundation (South Africa) and the Swedish Institute, Stockholm, Sweden.

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