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# **Fermentation of glucose-xylose-arabinose mixtures by a synthetic consortium of single-sugar-fermenting *Saccharomyces cerevisiae* strains**

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## Abstract

D-Glucose, D-xylose and L-arabinose are major sugars in lignocellulosic hydrolysates. This study explores fermentation of glucose-xylose-arabinose mixtures by a consortium of three 'specialist' *Saccharomyces cerevisiae* strains. A D-glucose- and L-arabinose-tolerant xylose specialist was constructed by eliminating hexose phosphorylation in an engineered xylose-fermenting strain and subsequent laboratory evolution. A resulting strain anaerobically grew and fermented D-xylose in the presence of 20 g L<sup>-1</sup> of D-glucose and L-arabinose. A synthetic consortium that additionally comprised a similarly obtained arabinose specialist and a pentose-non-fermenting laboratory strain, rapidly and simultaneously converted D-glucose and L-arabinose in anaerobic batch cultures on three-sugar mixtures. However, performance of the xylose specialist was strongly impaired in these mixed cultures. After prolonged cultivation of the consortium on three-sugar mixtures, the time required for complete sugar conversion approached that of a previously constructed and evolved 'generalist' strain. In contrast to the generalist strain, whose fermentation kinetics deteriorated during prolonged repeated-batch cultivation on a mixture of 20 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose and 5 g L<sup>-1</sup> L-arabinose, the evolved consortium showed stable fermentation kinetics. Understanding the interactions between specialist strains is a key challenge in further exploring the applicability of this synthetic consortium approach for industrial fermentation of lignocellulosic hydrolysates.

**Keywords:** bioethanol, mixed-culture fermentation, evolutionary engineering, pentose fermentation, yeast

## Background

Industrial production of fuel ethanol by *Saccharomyces cerevisiae* still predominantly relies on hydrolysed cane sugar and corn starch as carbon sources (Renewable Fuel Association, 2017). Alternatively, fermentable sugar mixtures can be generated by hydrolysis of agricultural residues such as corn stover, wheat straw, corn fiber or corn cobs (Lynd, 1996, van Maris *et al.*, 2006). Cost-effective operation of such processes, for which the first full-scale plants have recently come on line (Jansen *et al.*, 2017), requires complete conversion of all sugars in lignocellulosic hydrolysates. In addition to D-glucose, these hydrolysates contain substantial amounts of D-xylose (10-25% of dry biomass) and L-arabinose (usually 2-3%, although some hydrolysates contain up to 20% L-arabinose) (Grohmann & Bothast, 1994, Grohmann & Bothast, 1997, van Maris *et al.*, 2006).

Intensive metabolic engineering of *S. cerevisiae*, which cannot naturally ferment pentoses, has enabled efficient anaerobic fermentation of D-xylose and L-arabinose (van Maris *et al.*, 2006, Jansen *et al.*, 2017). In engineered strains, pentoses enter the yeast metabolic network as D-xylulose-5-phosphate, whose entry into glycolysis is facilitated by overexpression of the enzymes of the non-oxidative pentose-phosphate pathway (PPP) (Walfridsson *et al.*, 1995, Kuyper *et al.*, 2005, Brat *et al.*, 2009, Madhavan *et al.*, 2009, Dun, 2012, Hector *et al.*, 2013, Hou *et al.*, 2016). Conversion of D-xylose into D-xylulose-5-phosphate in these strains relies on overexpression of xylulokinase (Xks1), along with heterologous expression of a xylose isomerase (Kuyper *et al.*, 2003, Brat *et al.*, 2009, Madhavan *et al.*, 2009, Dun, 2012, Hector *et al.*, 2013, Hou *et al.*, 2016) or combined heterologous expression a xylose reductase and xylitol dehydrogenase (Kötter & Ciriacy, 1993, Hahn-Hägerdal *et al.*, 2001, Jeffries, 2006, Watanabe *et al.*, 2007,

Runquist *et al.*, 2010). Conversion of L-arabinose into D-xylulose-5-phosphate has been achieved by expression of bacterial genes encoding L-arabinose isomerase (AraA), L-ribulokinase (AraB), and L-ribulose-5-phosphate-4-epimerase (AraD) (Sedlak & Ho, 2001, Becker & Boles, 2003, Wisselink *et al.*, 2007), combined with deregulation of the Gal2 galactose permease, which also transports L-arabinose (Sedlak & Ho, 2001, Wisselink *et al.*, 2010, Subtil & Boles, 2011, Wang *et al.*, 2017). Further knowledge-based engineering, random mutagenesis and evolutionary engineering has yielded *S. cerevisiae* strains that are now applied at industrial scale for conversion of lignocellulosic hydrolysates (Lee *et al.*, 2012, Zhou *et al.*, 2012, Lee *et al.*, 2014, Crook *et al.*, 2016, Hou *et al.*, 2016)(for a recent review see (Jansen *et al.*, 2017)).

Anaerobic fermentation of sugar mixtures by pentose-fermenting *S. cerevisiae* strains is typically characterized by a fast phase of glucose fermentation, followed by slower utilization of the two pentoses (Bettiga *et al.*, 2009, Wisselink *et al.*, 2009, Wang *et al.*, 2017). Maximizing fermentation rates throughout mixed-sugar conversion processes will not only benefit volumetric productivity of industrial processes, but also increase tolerance to inhibitors present in lignocellulosic hydrolysates (Bellissimi *et al.*, 2009, Wright *et al.*, 2011, Jansen *et al.*, 2017). Poor kinetics of Hxt- and Gal2-mediated pentose transport, reflected by low affinity for these pentoses and strong competitive inhibition by glucose play a key role in the preferential use of glucose and 'tailing' of pentose concentrations towards the end of fermentation processes (Sonderegger & Sauer, 2003, Kuyper *et al.*, 2005, Wisselink *et al.*, 2009, Sanchez *et al.*, 2010). Intensive research is therefore directed at improving pentose-uptake kinetics by expressing variants of native Hxt transporters and/or heterologous transporters that enable high-affinity pentose uptake in the presence of glucose (Hamacher *et al.*, 2002, Sedlak & Ho, 2004, Runquist *et al.*, 2010, Subtil & Boles, 2011, Subtil & Boles, 2012, Farwick *et al.*, 2014,

Knoshaug *et al.*, 2015, Li *et al.*, 2015, Bracher *et al.*, 2018) as well as by improvement of the kinetics of pentose isomerases (Lee *et al.*, 2012, Lee *et al.*, 2014, Crook *et al.*, 2016). While progress has been made in optimizing pentose fermentation kinetics, engineered *S. cerevisiae* strains described in the public domain still exhibit lower fermentation rates on pentoses than on D-glucose (Jansen *et al.*, 2017).

So far, research on fermentation of lignocellulosic hydrolysates by *S. cerevisiae* has focused on development of 'generalist' yeast strains, capable of fermenting mixtures of D-glucose, D-xylose and L-arabinose. However, from a theoretical perspective, the maximum conversion rate under substrate-excess conditions can only be reached when, through evolutionary adaptation or strain engineering, a microbe preferentially allocates its cellular resources (e.g. ribosomal capacity, ATP, amino acids) to fast conversion of a single substrate (Berkhout *et al.*, 2013). This principle, which explains evolution of sequential (diauxic) substrate utilization during mixed-substrate utilization by wild-type micro-organisms, suggests that use of consortia of yeast strains specialized in the fermentation of either D-glucose, D-xylose or L-arabinose might enable better mixed-sugar fermentation kinetics than application of a single generalist strain.

An additional potential advantage of mixed-sugar conversion by consortia of specialist strains relates to process stability. To optimize volumetric productivity, industrial processes should ideally recycle yeast biomass, rather than to initiate each new batch cycle with a new, freshly propagated inoculum of yeast biomass. Such yeast biomass recycling requires stability of fermentation kinetics through a large number of cultivation cycles. However, laboratory evolution experiments with engineered pentose-fermenting generalist yeast strains have shown progressive degeneration of their pentose fermentation kinetics during prolonged growth in repeated batch cultures

(Wisselink *et al.*, 2009, Mans *et al.*, 2018). This observation has also been attributed to a strong selective pressure for resource allocation to a preferred substrate, at the expense of the utilization of less preferred substrates (Wisselink *et al.*, 2009, Mans *et al.*, 2018).

The trade-offs imposed by resource allocation and/or metabolic interference between different substrate-conversion pathways should, in theory, not apply during conversion of substrate mixtures by consortia of 'specialist' microbes that can each only convert a single substrate (Alper & Stephanopoulos, 2009, Hanly *et al.*, 2012). Several previous studies have investigated conversion of glucose-xylose mixtures by defined microbial consortia. A binary consortium of recombinant *E. coli* single-sugar specialists strains was shown to efficiently produce lactate from a mixture of xylose and glucose (Eiteman *et al.*, 2008, Eiteman *et al.*, 2009). Studies on the use of defined microbial consortia for ethanol production from sugar mixtures focused on co-cultivation of non-engineered glucose-fermenting microbes such as *S. cerevisiae* with naturally D-xylose consuming organisms such as *Scheffersomyces stipitis*, *E. coli* or *Zymomonas mobilis* in which glucose metabolism was inactivated (Alper & Stephanopoulos, 2009, Chen, 2011). Biosynthetic oxygen requirements (Laplace *et al.*, 1993), byproduct formation (Hanly *et al.*, 2012), sensitivity to phages and/or lower ethanol tolerance (Chandrakant & Bisaria, 1998, van Maris *et al.*, 2006, Chen, 2011) represent challenges in the industrial application of such non-*Saccharomyces* ethanologens. Additionally, previous studies on ethanol production from sugar mixtures by synthetic microbial consortia have not compared the long-term stability of mixed-sugar fermentation in cultures of single generalist strains and consortia of specialists.

The goal of the present study was to explore conversion of mixtures of D-glucose, D-xylose and L-arabinose by a synthetic consortium of a glucose-fermenting laboratory

strain of *S. cerevisiae* and two glucose-phosphorylation-deficient *S. cerevisiae* strains engineered and evolved for efficient fermentation of either D-xylose or L-arabinose in the presence of the other two sugars. After studying fermentation of sugar mixtures by the individual specialist strains, fermentation kinetics of the consortium were improved by laboratory evolution. Performance and stability of fermentation kinetics by the consortium during prolonged, anaerobic repeated batch cultivation were compared with that of a previously described 'generalist' strain engineered and evolved for fermentation of glucose-pentose mixtures (Wisselink *et al.*, 2009).

## Materials and Methods

**Strains and maintenance.** The *S. cerevisiae* strains used in this study were derived from the CEN.PK lineage (Entian & Kötter, 2007, Nijkamp *et al.*, 2012) (Table 1). For storage, strains were grown on synthetic medium (Verduyn *et al.*, 1992) containing 20 g L<sup>-1</sup> D-glucose or, in the case of glucose-phosphorylation-negative, xylose- or arabinose-fermenting strains, 20 g L<sup>-1</sup> D-xylose or 20 g L<sup>-1</sup> L-arabinose respectively. Auxotrophic strains were grown on yeast-extract/peptone (YP) medium (10 g L<sup>-1</sup> Bacto yeast extract (Becton Dickinson, Franklin Lakes, NJ) and 20 g L<sup>-1</sup> Bacto Peptone (Becton Dickinson). Single-colony isolates obtained after laboratory evolution were grown in synthetic medium containing 20 g L<sup>-1</sup> of each D-glucose, D-xylose and L-arabinose. After strains were grown in shake flasks (Verhoeven *et al.*, 2017), glycerol (30% vol/vol) was added and 1 mL aliquots were stored at -80 °C.

**Media and shake flask cultivation.** Synthetic medium (SM) and sugar solutions were prepared as described previously (Verduyn *et al.*, 1992). After autoclaving the mineral salts medium for 20 min at 121 °C, filter-sterilized vitamin solution (Verduyn *et al.*, 1992) and 50 % (w/w) sterile solutions of D-glucose, D-xylose and L-arabinose were



added. Prior to inoculation, 20 g L<sup>-1</sup> L-arabinose (SMA), 20 g L<sup>-1</sup> D-glucose (SMD), 20 g L<sup>-1</sup> D-xylose (SMX), 20 g L<sup>-1</sup> L-arabinose and 20 g L<sup>-1</sup> D-glucose (SMAG), 20 g L<sup>-1</sup> D-xylose and 20 g L<sup>-1</sup> D-glucose (SMXG), or 20 g L<sup>-1</sup> L-arabinose, 20 g L<sup>-1</sup> D-glucose and 20 g L<sup>-1</sup> D-xylose (SMAGX) were added to SM as carbon sources. Solid media were prepared by adding 20 g L<sup>-1</sup> agar (Becton Dickinson) to SM or YP medium prior to autoclaving at 121 °C for 20 min. Shake-flask cultures were conducted in 500-ml flasks containing 100 ml of medium and were incubated in an orbital shaker at 200 rpm set at 30 °C. Physiological characterization of aerobic growth was performed in shake flasks containing SMX or SMXG with urea as sole nitrogen source (Luttik *et al.*, 2000) to prevent acidification. Cultures were prepared by inoculating frozen stocks (1 ml aliquots in 30% glycerol) directly into pre-culture shake flasks. In late exponential phase an aliquot was transferred to a second pre-culture to obtain an initial OD<sub>660</sub> of 0.1. All cultures used for physiological characterization were inoculated from such second pre-cultures, growing in late exponential phase. Shake-flask cultures grown under anaerobic conditions were incubated at 30 °C in an Innova anaerobic chamber (5% H<sub>2</sub>, 6% CO<sub>2</sub>, and 89% N<sub>2</sub>, New Brunswick Scientific, Edison, NJ) in 50 mL shake flasks placed on an orbital shaker set at 200 rpm. synthetic media used for anaerobic cultivations were supplemented with the anaerobic growth factors Tween 80 (420 mg L<sup>-1</sup>) and ergosterol (10 mg L<sup>-1</sup>), dissolved in ethanol (Verduyn *et al.*, 1990). To avoid growth limitation by anaerobic growth factors at biomass concentrations above 2.5 g L<sup>-1</sup> (Verduyn *et al.*, 1990), Tween 80 and ergosterol concentrations in culture stability experiments were increased to 504 mg L<sup>-1</sup> and 12 mg L<sup>-1</sup> respectively.

**Strain construction.** *S. cerevisiae* strains were transformed following the protocol of Gietz and Woods (Gietz *et al.*, 1995). The plasmids used in this study are listed in Additional File 1. Plasmid DNA was isolated from *E. coli* cultures using a GenElute

Plasmid kit (Sigma-Aldrich, St. Louis, MO). Nine DNA fragments carrying the expression cassettes of the *Piromyces* SP E2. *xylA* and a single overexpression cassette of *xks1* were PCR amplified from pUD350 and pUD353 (Verhoeven *et al.*, 2017) using the primers as listed in Additional File 2. The PCR amplifications added homologous flanks that facilitated *in vivo* assembly and integration into the *CAN1* locus. Strain IMX604 was co-transformed with all ten fragments and the *CAN1*-gRNA plasmid pMEL10 (Mans *et al.*, 2015). Transformed cells were incubated for one hour in SMD after which they were plated on SMX. Colonies were restreaked thrice on SMX plates and correct assembly of all ten fragments in the *CAN1* locus was confirmed by diagnostic PCR (Dreamtaq, Thermo Scientific). Plasmid pMEL10 was counter selected on YP with 20 g L<sup>-1</sup> D-xylose (YPX) agar with 5-fluoroorotic acid (5-FOA) as described previously (Mans *et al.*, 2015). *HXX2* was deleted in the resulting strain IMX659 by co-transforming plasmid pUDE327 and the *PcaraT* expression cassette obtained from pPWT118 as the repair fragment. After counter selection of pUDE327 with 5-FOA, the wild-type *URA3* gene was restored as described previously (Mans *et al.*, 2015), yielding strain IMX730.

**Batch cultivation and laboratory evolution.** Anaerobic batch cultivation was performed in 2-L laboratory bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 L, which were stirred at 800 rpm and continuously sparged with nitrogen gas (<10 ppm oxygen) at 0.5 L min<sup>-1</sup>. Temperature was set at 30 °C and culture pH was controlled at 5.0 by automated addition of 2 M KOH. To minimize oxygen diffusion, anaerobic bioreactors were equipped with Viton O-rings and Norprene tubing (Cole Palmer Instrument Company, Vernon Hills, IL). Excessive foaming was prevented by adding 0.2 g L<sup>-1</sup> antifoam C (Sigma-Aldrich, St. Louis, MO) to synthetic media used for bioreactor cultivation. Precultures were pelleted by centrifugation and resuspended in demi water prior to inoculation. Laboratory

evolution experiments for improving D-xylose fermentation in the presence of D-glucose and L-arabinose, as well as culture stability experiments were performed in sequential batch reactors (SBRs). On-line measurement of CO<sub>2</sub> concentrations in the off gas of SBRs was used as input for a control routine programmed in MFCS/win 3.0 (Sartorius AG, Göttingen, Germany). During each cycle, an empty-refill cycle was automatically initiated when the CO<sub>2</sub> concentration in the exhaust gas had first increased above a threshold value of 0.2% (indicating growth) and subsequently decreased below a second threshold of 0.1 % (indicating sugar depletion). For culture stability experiments, the latter threshold was set at 0.05% as this value approximately corresponded to the CO<sub>2</sub> output of a non-growing culture. After the emptying phase, when approximately 7% of the initial culture volume was left in the reactor, the reactor was automatically refilled with fresh medium from a 20-L glass vessel, which was continuously sparged with nitrogen gas. Single colony isolates were obtained by three consecutive restreaks using samples of the single strain laboratory evolution cultures on SMAGX agar plates, incubated anaerobically at 30 °C. Laboratory evolution of the consortia was initiated in 50 mL shake-flasks containing SMAGX with urea to prevent acidification. Synthetic medium used for IMS0010 fermentations, consortium cultivation and culture stability experiments in SBRs were supplemented with twice the amount of vitamins solution to avoid nutrient limitations. Moreover, additional Tween-80 and ergosterol (504 mg L<sup>-1</sup> and 12 mg L<sup>-1</sup> respectively) were added when the biomass concentration in these cultures reached 2.5 g dw L<sup>-1</sup>. The initial concentrations of these anaerobic growth factors were not altered, in order to avoid growth inhibition (Verduyn *et al.*, 1990).

**Analytical methods.** Biomass optical density measurements at 660nm were performed with a Libra S11 spectrometer (Biochrom, Cambridge, United Kingdom). Specific

growth rates were calculated based on biomass dry weight measurements performed on at least six samples taken during the exponential growth phase. Culture dry weight (CDW) was analysed by filtering 10 mL culture samples over pre-weighed nitrocellulose filters (pore size, 0.45  $\mu\text{m}$ ; Gelman Laboratory, Ann Arbor, MI). Filters were washed with demineralized water and dried in a microwave oven (Bosch, Stuttgart, Germany) for 20 min at 360 W. Bioreactor exhaust gas was cooled by a condenser (2 °C) and dried with a Permapure MD-110-48P-4 dryer (Permapure, Toms River, NJ). CO<sub>2</sub> concentrations in the dried gas were measured using an NGA 2000 analyser (Rosemount Analytical, Orrville, OH). Metabolite concentrations in culture samples were determined by centrifugation and subsequent analysis of the supernatant by high-performance liquid chromatography (HPLC) on an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a Bio-Rad HPX 87 H column (Bio-Rad, Hercules, CA) eluted at 60 °C with 0.5 g L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml min<sup>-1</sup>. Metabolite levels were quantified using an Agilent G1362A refractive-index detector and an Agilent G1314F VWD detector. D-xylitol concentrations in the presence of L-arabinose were measured using a D-sorbitol/xylitol assay kit (Megazyme International Ireland, Wicklow, Ireland). Correction for ethanol evaporation was done for all bioreactor experiments as described previously (Guadalupe Medina *et al.*, 2010).

## Results

**Repeated batch cultivation of a pentose-fermenting *S. cerevisiae* strain on sugar mixtures leads to deterioration of fermentation kinetics.** The D-glucose, D-xylose and L-arabinose fermenting *S. cerevisiae* strain IMS0010 was previously generated by a combination of metabolic and evolutionary engineering (Wisselink *et al.*, 2009). Evolutionary engineering of this strain involved prolonged cultivation in sequential

batch reactors (SBRs) that were alternately grown on SMA, SMAG and SMAGX. This dynamic cultivation regime was designed to avoid selection for faster fermentation of glucose at the expense of pentose fermentation kinetics (Wisselink *et al.*, 2009). In anaerobic batch cultures, strain IMS0010 first consumed D-glucose and only then the two pentose sugars (Figure 1A, (Wisselink *et al.*, 2009)).

To investigate stability of the mixed-sugar fermentation kinetics of *S. cerevisiae* IMS0010 during repeated batch cultivation on a mixture of three sugars, duplicate anaerobic SBR cultures were performed. To resemble sugar concentrations in common lignocellulosic hydrolysates (van Maris *et al.*, 2006), these cultures were grown on SM supplemented with 20 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose and 5 g L<sup>-1</sup> L-arabinose. Over the first 6 cycles (ca. 200 h of cultivation), carbon dioxide production profiles revealed stable fermentation kinetics (Figure 1A, Additional File 3A). After this time, the length of the SBR cycles progressively increased (Figure 1B). In one of the reactors, a mechanical failure, which occurred after 600 h, resulted in premature execution of the empty-refill routine during the glucose consumption phase. This incident coincided with a sharp increase of the cycle length in the next cycle, from which the culture did not recover (Additional File 3B). After 38 days of operation, the cycle time of both reactors had increased from 25 ± 0.6 h in the fifth cycle to 51 ± 1.2 h in the final cycle (24<sup>th</sup> and 27<sup>th</sup> cycle for reactor 1 and 2, respectively). Biomass and extracellular metabolite measurements, analysed during the fifth and final SBR cycles, indicated that slower conversion of the sugar mixture was primarily due to deterioration of L-arabinose fermentation kinetics (Figures 1A and 1C).

**Construction of a D-xylose fermenting specialist strain.** Glucose-phosphorylation-negative, D-xylose-metabolizing *S. cerevisiae* strains have previously been constructed

and applied for *in vivo* evolution of Hxt transporter variants that enable D-xylose uptake in the presence of high glucose concentrations (Farwick *et al.*, 2014, Nijland *et al.*, 2014, Wisselink HW *et al.*, 2015). However, anaerobic growth of such glucose-phosphorylation-negative strains on D-xylose has not previously been studied. To construct a 'xylose-specialist' *S. cerevisiae* strain, multiple copies of an expression cassette for *Piromyces xylA* and a single copy of a *XKS1* overexpression cassette were introduced in strain IMX604 (*hxx1Δ glk1Δ gal1Δ::Spcas9-AmdSYM gre3Δ::NPPP*), yielding strain IMX659 (*hxx1Δ glk1Δ gal1Δ::Spcas9-AmdSYM gre3Δ::NPPP can1Δ::xylA\*9-XKS1*) (Additional File 4). After disrupting the remaining hexokinase gene *HXX2* by integration of an expression cassette for the high-affinity *PcAraT* transporter (Bracher *et al.*, Verhoeven *et al.*, 2018) a functional *URA3* gene from *S. cerevisiae* CEN.PK113-7D was introduced, yielding the prototrophic xylose specialist strain IMX730 (*hxx1Δ glk1Δ gal1Δ::Spcas9-AmdSYM gre3Δ::NPPP can1Δ::xylA\*9-XKS1 hxx2Δ::PcAraT*). In aerobic shake-flask cultures on SMX, strain IMX730 exhibited a specific growth rate of 0.22 h<sup>-1</sup> (Figure 2A) while no growth was observed on SMD or SMA. In agreement with previous reports on inhibition of D-xylose by glucose (Farwick *et al.*, 2014, Nijland *et al.*, 2014), a lower specific growth rate was observed on SMXG (0.16 h<sup>-1</sup>).

Despite the ability of strain IMX730 to grow aerobically on D-xylose in the presence of D-glucose, 2000 h of incubation in duplicate anaerobic bioreactors containing SMAGX did not result in observable growth (Additional File 5). After this long incubation period, a limiting oxygen feed (headspace aeration with 0.5 L min<sup>-1</sup> of air while stirring the bioreactor at 300 rpm) was applied to generate active biomass. Subsequently, the two reactors were partially emptied, refilled with fresh medium containing only D-xylose and switched back to fully anaerobic conditions. When growth

was observed after 16 days, the culture was switched to SBR mode. During the first four cycles, the initial concentrations of D-xylose and L-arabinose were kept at 20 g L<sup>-1</sup>, while the concentration of D-glucose was incrementally increased from 0 g L<sup>-1</sup> to 20 g L<sup>-1</sup>. Subsequently, over 150 days of SBR cultivation on SMAGX, the specific growth rate, as estimated from CO<sub>2</sub> production profile, progressively increased to ca. 0.14 h<sup>-1</sup> and then stabilized (Figure 2b). After 254 and 284 days (Reactors 1 and 2, respectively), single colony isolates were obtained by plating and anaerobic incubation on SMAGX agar. The specific growth rates of 8 isolates from each reactor were analysed in anaerobic shake-flask cultures on SMAGX (Additional File 6). Based on these experiments, xylose specialist strains IMS0535 and IMS0537, which were selected from different SBR experiments, were grown on SMAGX in anaerobic batch reactors (Additional File 7). Their estimated specific growth rates (0.13 h<sup>-1</sup> and 0.12 h<sup>-1</sup>, respectively) in these anaerobic cultures closely resembled those of the evolved populations at the end of the SBR evolution experiments from which they originated (Additional File 5).

**Anaerobic fermentation of mixtures of D-glucose, D-xylose and L-arabinose by individual 'specialist strains'.** To provide a baseline for interpretation of experiments with consortia of specialist strains, the D-xylose specialist strain IMS0535 described above and the 'glucose specialist' laboratory reference strain CEN.PK113-7D (Entian & Kötter, 2007) were characterized in separate anaerobic batch reactors containing synthetic medium with 20 g L<sup>-1</sup> D-glucose, 20 g L<sup>-1</sup> D-xylose and 20 g L<sup>-1</sup> L-arabinose (Figure 3A and 3C). Data from a published study on the hexose-phosphorylation-deficient L-arabinose specialist strain IMS0522 (Verhoeven *et al.*, 2018) are shown in Figure 3B. Sugar consumption profiles confirmed that each of the specialist strains only

consumed a single sugar (Figure 3). The glucose and arabinose specialists reached complete sugar conversion after 12 and 24 h, respectively. Conversion of D-xylose by strain IMS0535 was slower and, when the D-xylose concentration decreased below ca. 5 g L<sup>-1</sup>, its conversion rate decelerated, leaving ca. 1 g L<sup>-1</sup> of residual D-xylose after 50 h (corresponding to 95 % conversion). The D-xylose consumption rate of strain IMS0535 in anaerobic cultures on SMAGX (Table 2) was approximately 50% lower than the highest reported rates of xylose consumption reported for engineered *S. cerevisiae* strains grown in synthetic medium with D-xylose as the only sugar (Zhou *et al.*, 2012, Jansen *et al.*, 2017).

When biomass-specific conversion rates of each of the three sugars during anaerobic growth of the specialist strains on SMAGX were compared with those of the generalist strain IMS0010 (Table 2), the specialist strains consistently showed higher conversion rates. However, based solely on the single-strain experiments of the current xylose-specialist IMS0535 (Figure 3A), a consortium of these three specialists inoculated at similar cell densities would not be expected to reduce the total fermentation time for anaerobic growth on SMAGX relative to strain IMS0010.

**Suboptimal conversion of sugar mixtures by a consortium of specialist strains.** To investigate the impact of co-cultivation of the three specialist strains on fermentation kinetics, two anaerobic bioreactor batch cultures, inoculated with 0.36 g biomass consisting of equal amounts of biomass of strains IMS0535, IMS0522 and CEN.PK113-7D were grown on SMAGX (Figure 4A). For comparison, growth and fermentation kinetics of the ‘generalist’ strain IMS0010 were characterised under the same conditions (Figure 4C).



During anaerobic co-cultivation of the consortium on SMAGX, D-glucose and L-arabinose were consumed simultaneously and completely (Figure 4A). Sugar consumption kinetics and, consequently, the time required for full conversion of these sugars resembled those observed in the corresponding single-culture experiments (Figure 3). In contrast, D-xylose fermentation kinetics of the consortium strongly differed from those observed in the single-strain experiment with the xylose specialist IMS0535 (Figure 2B). In the mixed culture, only 15% of the available D-xylose was consumed within 50 h, indicating that growth and/or fermentation kinetics of IMS0535 were severely inhibited by the presence of the D-glucose and/or L-arabinose specialists. Similar mixed-culture fermentation kinetics were observed when strain IMS0535 was replaced by the independently evolved xylose-specialist strain IMS0537 (Additional File 8).

Extracellular metabolite measurements showed higher concentrations of D-xylitol (up to 0.6 g L<sup>-1</sup>) in cultures of consortia containing the xylose specialist strain IMS0535 than in pure cultures of this strain grown on SMAGX (Additional File 9). *In vitro* experiments have shown that D-xylitol is a competitive inhibitor of the *Piromyces* xylose isomerase (Brat *et al.*, 2009, Lee *et al.*, 2017). However, anaerobic shake-flask cultivation of strain IMS0535 on SMAGX supplemented with 1.5 g L<sup>-1</sup> of D-xylitol, which is 2.5-fold higher than the concentration observed in the mixed cultures, showed a growth rate decrease of less than 10%, while 80% consumption of D-xylose was reached within 50 h (Additional File 10). Acetate concentrations were slightly higher than in cultures of the pentose fermenting strain IMS0010 (Additional File 9). Increasing the inoculum size of the xylose-specialist strain IMS0535 relative to that of the other specialists resulted in a slight improvement of D-xylose fermentation kinetics, but D-

xylose conversion still strongly decelerated as fermentation of D-glucose and L-arabinose progressed (Figure 4B).

**Laboratory evolution of a consortium of specialist strains for improved fermentation of sugar mixtures.**

To improve kinetics of mixed-sugar fermentation by the consortium, and especially its D-xylose fermentation kinetics, anaerobic laboratory evolution experiments were performed (Figure 5A). These experiments were started in anaerobic shake-flask cultures of the consortium (strains IMS0535, IMS0522 and CEN.PK113-7D) on SMAGX. The initial shake-flask cultures showed the same slow D-xylose fermentation previously observed in anaerobic bioreactors (Figure 4B). When 2 mL samples from stationary-phase shake-flask cultures on SMAGX were used to inoculate fresh shake flasks on the same medium, D-glucose and L-arabinose were completely consumed within 2 days, whereas complete conversion of D-xylose took 3 weeks. Subsequent transfer to fresh SMAGX showed full conversion of D-glucose and D-xylose within 4 days. However, L-arabinose was not converted within this time span, possibly because cells of the arabinose specialist did not survive prolonged starvation. Therefore, at the fourth transfer, the arabinose specialist IMS0522 was reintroduced by supplementing 1 mL of a stationary-phase cultures of this strain on SMAGX, after which the consortium converted all three sugars within 4 days. Subsequently, repeated batch cultivation was continued in duplicate anaerobic SBR cultures on SM + 10 g L<sup>-1</sup> each of L-arabinose, D-glucose and D-xylose (Figure 5A). Already during the fifth cycle, all three sugars were fully consumed within 31 h (Figure 5D).

After 21 cycles of SBR cultivation (680 h and 750 h for the two reactors), the composition of the sugar mixture was changed to 20 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose

and 5 g L<sup>-1</sup> L-arabinose, which resembles the relative concentrations of the three sugars in lignocellulosic hydrolysates (van Maris *et al.*, 2006). Off-gas CO<sub>2</sub> profiles indicated that, for cultures of the consortium, this change strongly affected fermentation kinetics, especially after the initial fast consumption of D-glucose (Additional File 11). As a result, the SBR cycle time increased from 33 ± 2 h to 47 ± 2 h. When SBR cultivation on the adapted sugar mixture was continued for a further 1200 h, the cycle length progressively decreased until all three sugars were consumed within 35 ± 1 h (Figure 5E and Additional File 11).). Subsequently, fermentation kinetics of both SBR cultures remained stable for an additional 1000 h (Figure 5C).

The improvement of the fermentation performance and subsequent stable fermentation kinetics of the consortium (Figure 5) provided a marked contrast with the deteriorating pentose fermentation kinetics of the generalist strain IMS0010 during prolonged SBR cultivation on SMAGX (Figure 1). However, the sugar compositions for the first 20 cycles of the SBR experiments with the consortium (Figure 5) were different from those in the SBR experiments with strain IMS0010 (Figure 1). Therefore, additional duplicate SBR experiments were performed in which the generalist strain IMS0010 was first grown for 21 cycles on 10 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose and 10 g L<sup>-1</sup> L-arabinose. During this phase, no marked deterioration of its fermentation kinetics was observed (Additional File 13A and B). When both SBRs were subsequently switched to a medium containing 20 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose and 5 g L<sup>-1</sup> L-arabinose, the overall fermentation cycle duration initially stayed the same. However, already after the fifth cycle of repeated batch cultivation, fermentation kinetics started to deteriorate (Additional File 13C). Metabolite analyses showed that these deteriorated sugar consumption kinetics were due to a slower consumption of both D-xylose and L-arabinose (Additional File 13D). In the second reactor, a sharper deterioration of sugar

fermentation kinetics occurred upon an interruption of the automated pH control. Although growth and sugar consumption resumed after this discontinuity, fermentation kinetics remained slower than before the perturbation (Additional File 14).

## Discussion

**Instability of a generalist pentose-fermenting yeast strain during repeated batch cultivation.** Prolonged SBR cultivation of the previously described ‘generalist’ pentose-fermenting strain *S. cerevisiae* IMS0010 (Wisselink *et al.*, 2009) on sugar mixtures whose relative concentrations of D-glucose, D-xylose and L-arabinose resembled those in lignocellulosic hydrolysates, led to progressive deterioration of fermentation kinetics (Figure 1). This deterioration was caused by decreasing pentose fermentation rates after an initial, fast phase of D-glucose. This observation indicates that repeated batch cultivation of this generalist strain on sugar mixtures favoured specialization towards fast utilization of glucose, at the expense of pentose fermentation. Selection for such specialization could reflect competition for limited cellular resources (Wisselink *et al.*, 2009), for example due to the need for high-level expression of heterologous isomerases for efficient pentose fermentation (Zhou *et al.*, 2012, Demeke *et al.*, 2015, Verhoeven *et al.*, 2017). Alternatively or additionally, negative interactions between proteins, metabolites, cofactors and effectors of the catabolic pathways for the three sugars may generate a selective pressure for specialization towards the fast use of a single sugar.

During repeated batch cultivation on mixed substrate, the cumulative selective pressure for fast utilization of each individual substrate is proportional to the number of generations of selective growth on that substrate. When, in such mixed substrate

cultures, sugars are consumed sequentially, this number of generations is strongly influenced by their order of consumption as well as by their relative concentrations in the growth medium. In line with the latter factor, deterioration of fermentation kinetics was much more pronounced during prolonged SBR cultivation of strain IMS0010 on a medium in which the concentration of glucose was higher than that of the two pentoses (Figure 1) than in similar experiments in which the concentrations of the three sugars were equal (Additional File 13A and B).

In two independent, long-running SBR experiments on sugar mixtures, a rapid deterioration of pentose fermentation kinetics of the generalist strain coincided with perturbations resulting from technical malfunctions (Additional File 3 and 14). In both instances the negative selective pressure was already evident from an increasing cycle duration prior to the perturbation but was greatly augmented afterwards. This observation suggested that cellular stress associated with these events enriched for mutations that were already present in the population. The fast deterioration of the generalist strain IMS0010 after perturbations may have been partially caused by changes in copy number of the plasmids that, in this strain, were used for expression of genes involved in xylose- and arabinose metabolism. For the experiment in which pH control was temporarily interrupted, this enhanced deterioration may be related to the observation that a combination of low pH and presence of acetate more strongly affects anaerobic growth of engineered *S. cerevisiae* on D-xylose than on D-glucose (Bellissimi *et al.*, 2009). Evolution towards a more specialized phenotype, augmented by occasional process perturbations, as observed here during prolonged SBR cultivation of *S. cerevisiae* IMS0010, is likely to represent a major challenge for development of industrial 'generalist' strains that retain optimal fermentation kinetics through a large number of biomass recycling steps on lignocellulosic hydrolysates.

**Fermentation of sugar mixtures by a consortium of engineered, specialist yeast strains.** In anaerobic batch cultures grown on SMAGX (Figure 3), specific sugar consumption rates of pure cultures of a hexose-phosphorylation-deficient D-xylose-specialist strain, a similar L-arabinose specialist strain (Verhoeven *et al.*, 2018) and a pentose-non-fermenting 'glucose-specialist' laboratory strain were 60 to 280 % higher than the corresponding sugar-conversion rates of the generalist strain IMS0010 (Table 2). This difference, which was observed despite the constant presence of potentially inhibiting concentrations of non-fermentable sugars (Farwick *et al.*, 2014, Nijland *et al.*, 2014, Wisselink HW *et al.*, 2015, Verhoeven *et al.*, 2018) in batch cultures of the specialist strains, underlines the potential benefit of synthetic consortia for mixed substrate conversion. However, in contrast to the specialist strains, the generalist strain could use the biomass formed during glucose consumption for subsequent conversion of the pentoses (Figure 4C), thereby improving volumetric sugar consumption rates. In cultures on sugar mixtures, this benefit of the generalist strain offset the higher biomass-specific conversion rates of the xylose specialist strain, which was the slowest growing of the three specialists (Figures 3).

Co-cultivation of the three specialist strains (IMS0522, IMS0535 and CEN.PK113-7D) on a mixture of L-arabinose, D-glucose and D-xylose showed that mixed-culture performance could not be accurately predicted from the growth and fermentation kinetics of the individual strains in pure cultures (Figure 4A). In particular, fermentation kinetics of the xylose specialist strain were severely impaired when grown together with the other two specialist strains. A possible explanation for this inhibition is that (by-)products of the arabinose and glucose specialists inhibited D-xylose

fermentation. Acetate and D-xylitol have been shown to inhibit fermentation rates of xylose-fermenting *S. cerevisiae* strains and *in vitro* xylose isomerase activity, respectively (Bellissimi *et al.*, 2009, Brat *et al.*, 2009, Lee *et al.*, 2017). While both compounds were present at the end of the mixed-culture experiments, their concentrations remained well below 1 g L<sup>-1</sup> (Additional File 9). At a pH of 5.0 this concentration of acetate is unlikely to strongly affect xylose fermentation rates (Bellissimi *et al.*, 2009, González-Ramos *et al.*, 2016). Impaired D-xylose consumption can likewise not solely be attributed to D-xylitol accumulation, as shake-flask cultivations of IMS0535 with even higher D-xylitol concentrations (1.5 g L<sup>-1</sup>) only showed a minor impact on D-xylose fermentation kinetics (Additional File 10). This is in line with the previously observed discrepancy between the small impact of D-xylitol on D-xylose fermentation by a *xylA*-based *S. cerevisiae* strain and the strong inhibition of xylose isomerase measured *in vitro* (Ha *et al.*, 2011). The increased D-xylitol concentrations may, however, reflect a cellular stress response as its production from D-xylose is catalyzed by the stress-induced NADPH-dependent aldo-keto reductases encoded by *GCY1*, *YPR1*, *GRE3*, *ARA1*, *YJR096W* and *YDL124W* (Träff *et al.*, 2002, Chang *et al.*, 2007).

Although growth media were designed to prevent nutrient limitations, the sluggish D-xylose fermentation kinetics of the mixed cultures might still reflect competition of the three strains for one or more essential nutrients. In pure cultures, intracellular stores formed by excessive 'luxury uptake' (Boender *et al.*, 2011, Paalme *et al.*, 2014) at the start of a batch culture will be distributed over the growing population as cells divide. In contrast, in consortia, rapid uptake of key nutrients by faster growing partners could constrain the ability of slower growing strains to completely convert their substrate. This hypothesis is consistent with the positive impact of a larger

inoculum of the slower growing xylose specialist on overall D-xylose conversion (Figure 4B).

Laboratory evolution of mixed cultures of the D-glucose, D-xylose and L-arabinose fermenting specialist yeast strains eventually yielded a consortium that stably converted mixtures of the three sugars (Figure 5E). In contrast to prolonged SBR cultivation of strain IMS0010, long-term cultivation of the evolved consortia in SBR cultures resulted in stable fermentation kinetics (Figure 5C). This result was entirely consistent with the key hypothesis tested in this study that the fermentation kinetics of consortia of specialist strains are more robust during long-term cultivation on sugar mixtures in long-term cultures than a generalist strain.

## Conclusions

This study represents a first exploration of the conversion of mixtures of glucose, xylose and arabinose by a consortium of three 'specialist' *S. cerevisiae* strains. The conclusion that generalist pentose-fermenting strains are likely to be inherently unstable in terms of mixed-sugar fermentation kinetics during repeated batch cultures has important implications for strain optimization and industrial process design. The potential benefit of re-using biomass through multiple cycles of cultivation is illustrated by its large-scale use in Brazilian 'first-generation' bioethanol processes grown on sucrose as the carbon source, where it has even been shown to lead to selection for better performing strains (Gombert & van Maris, 2015). Our results show that, in terms of strain stability, use of consortia of specialist yeast strains for second-generation bioethanol could confer similar benefits, including an ability to adapt to fluctuations in feedstock composition. Moreover, unlike repeated batch cultivation of generalist strains such as strain IMS0010, repeated batch cultivation of consortia of specialist strains on actual



industrial hydrolysates may be used to select for tolerance to fermentation inhibitors, without the inherent risk of selecting for faster glucose fermentation at the expense of pentose fermentation kinetics. However, before industrial implementation can be contemplated, a deeper insight into the interaction between specialist strains and a further improvement of their sugar fermentation kinetics in mixed-culture processes is essential. Additionally, the consortium of glucose- and pentose-fermenting specialist yeast strains described in this study provides an interesting model to study the molecular ecology of synthetic consortia of industrial microbes. While outside the scope of the present study, resequencing of the genomes of the evolved strains, including those evolved as part of three-partner consortia and evaluation of the impact of the observed mutations by their reverse engineering into non-evolved strains (Oud *et al.*, 2012, Mans *et al.*, 2018), represents a logical next step in such research.

### List of abbreviations

**SM**: synthetic medium; **SMX**: synthetic medium with 20 g L<sup>-1</sup> D-xylose; **SMA**: synthetic medium with 20 g L<sup>-1</sup> L-arabinose; **SMXG**: synthetic medium with 20 g L<sup>-1</sup> D-xylose and 20 g L<sup>-1</sup> D-glucose; **SMAG**: synthetic medium with 20 g L<sup>-1</sup> L-arabinose and 20 g L<sup>-1</sup> D-glucose; **SMAGX**: synthetic medium with 20 g L<sup>-1</sup> L-arabinose, 20 g L<sup>-1</sup> D-glucose and 20 g L<sup>-1</sup> D-xylose; **YP**: yeast-extract/peptone; **OD**: optical density; **NPPP**: non-oxidative branch of the pentose-phosphate pathway.

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## **Competing interests**

DSM markets technology for biofuels production from lignocellulosic feedstocks, holds IP positions in this field and co-funded the research described in this publication.

## **Authors' contributions**

M.D.V., J.M.D., A.J.A.v.M. and J.T.P. together designed this study; M.D.V designed and performed all wet-lab experiments; S.d.V contributed to characterizing the co-cultivation experiments; M.D.V. and J.T.P. wrote the manuscript. All authors read and commented a draft version of the manuscript and approved the submitted version.

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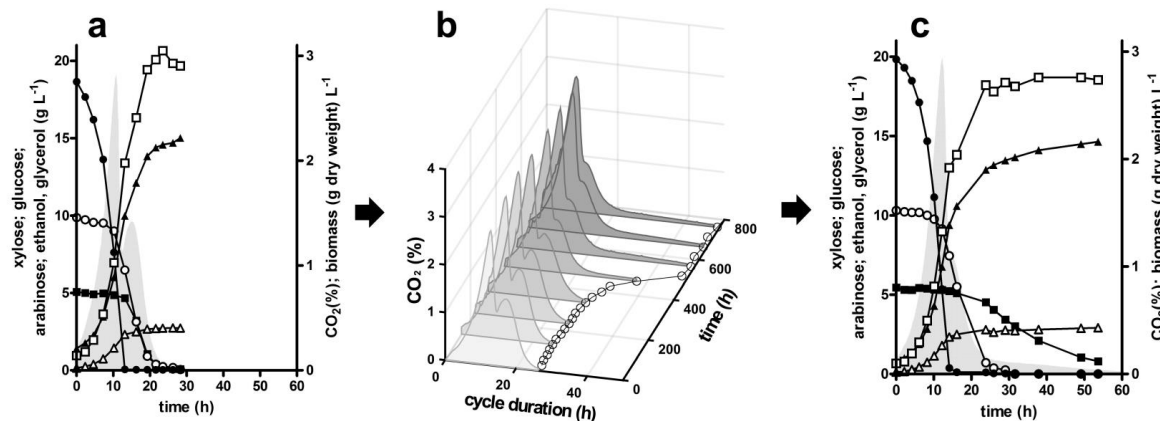
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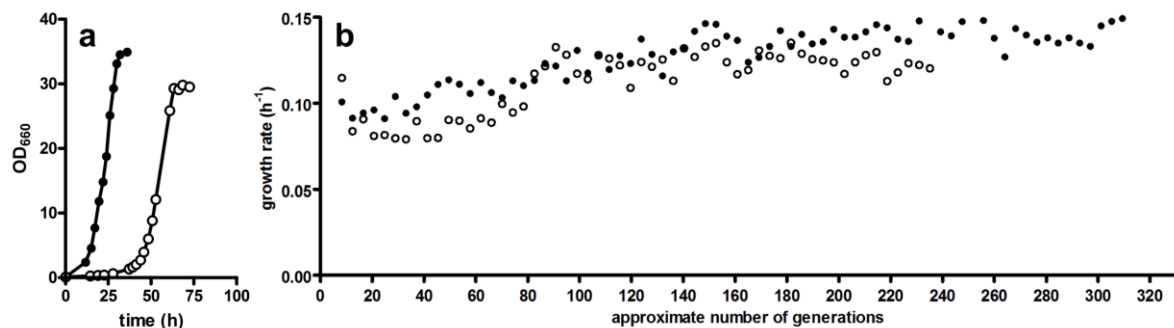
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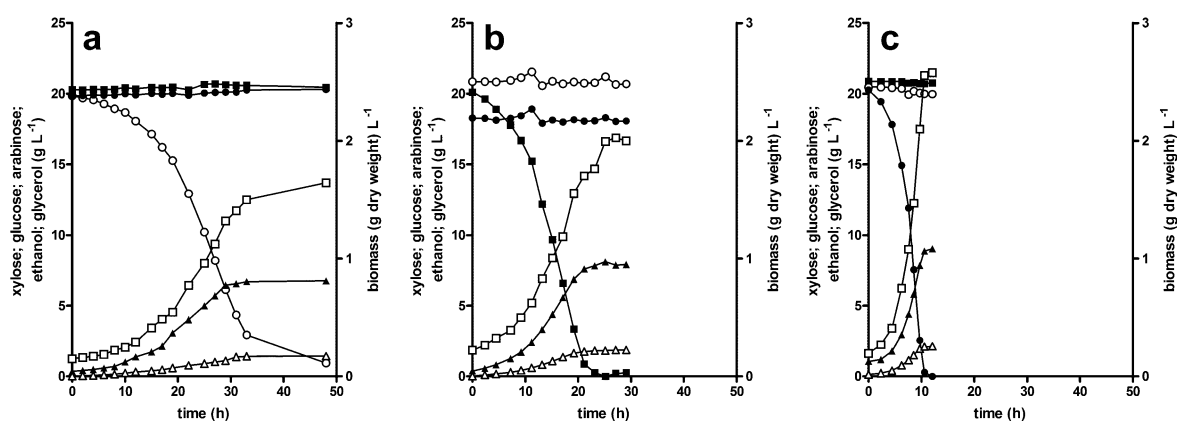


**Figure 1** | Metabolite concentrations, CO<sub>2</sub> production curves and cycle length (h) of an anaerobic sequential batch reactor experiment of the glucose-xylose-arabinose consuming generalist strain *S. cerevisiae* IMS0010 on synthetic medium containing 20 g L<sup>-1</sup> D-glucose, 10 g L<sup>-1</sup> D-xylose and 5 g L<sup>-1</sup> L-arabinose. Metabolite and CO<sub>2</sub> production profiles (*solid grey areas*) shown in **a.** and **c.** correspond to the fifth and 24<sup>th</sup> cycle of the SBR experiment. Symbols List of symbols: ● D-glucose, ○, D-xylose, ■, L-arabinose, □, biomass dry weight, ▲, ethanol, △, glycerol. **b.** 3-axis plot showing cycle length (○) and off-gas CO<sub>2</sub> profiles during prolonged SBR cultivation. To facilitate interpretation, this panel shows data for every fifth cycle 1, 5, 10, 15, etc. Complete off-gas CO<sub>2</sub> profiles for this experiment and an independent duplicate SBR experiment are provided in Additional File 3.

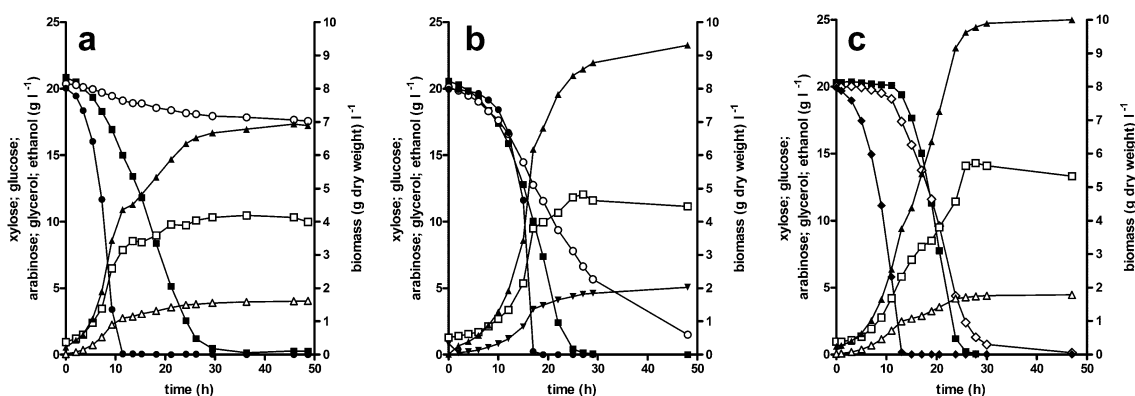




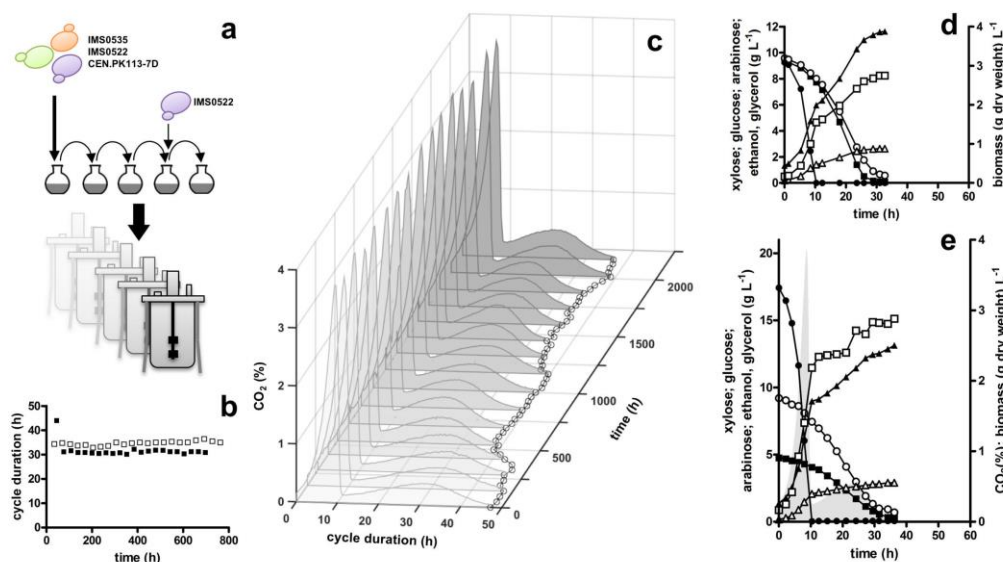
**Figure 2** | Growth and laboratory evolution of the xylose specialist strain *S. cerevisiae* IMX730 (*hvk1Δ glk1Δ gal1Δ::Spcas9-AmdSYM gre3Δ::NPPP hvk2Δ::PcaraT can1Δ::xylA\*9-XKS1*). **a.** Growth curves in aerobic shake flask cultures on synthetic medium with 20 g L<sup>-1</sup> D-xylose (●) or D-glucose and D-xylose (20 g L<sup>-1</sup> each, ○). Data shown are derived from one of two duplicate growth experiments, of which kinetic parameters differed by less than 5%. **b.** Specific growth rate on xylose (h<sup>-1</sup>) during two independent laboratory evolution experiments estimated from CO<sub>2</sub> production profiles in SBR reactors: SBR 1 (○) and SBR 2 (●), in synthetic medium with 20 g L<sup>-1</sup> L-arabinose, 20 g L<sup>-1</sup> D-xylose and an [increasing concentration of D-glucose \(up to 20 g L<sup>-1</sup>\)](#) in independent biological replicates. The first data point for each experiment corresponds to the initial aerobic batch culture, all subsequent values were obtained under anaerobic conditions. CO<sub>2</sub> off-gas profiles [and measured D-glucose glucose concentrations for](#) both SBR experiments are shown in Additional File 5.



**Figure 3** | Growth and extracellular metabolite concentrations in anaerobic batch cultures of the evolved pentose consuming, glucose-phosphorylation negative *S. cerevisiae* strains **a.** IMS0535 (D-xylose fermenting), **b.** IMS0522 (L-arabinose fermenting, data previously obtained (Verhoeven *et al.*, 2018)) and **c.** of the D-glucose fermenting laboratory strain CEN.PK113-7D. All cultures were inoculated to a concentration of 0.12 g biomass dry weight L<sup>-1</sup> in anaerobic bioreactors containing synthetic medium with 20 g L<sup>-1</sup> D-glucose, 20 g L<sup>-1</sup> D-xylose and 20 g L<sup>-1</sup> L-arabinose. List of symbols: ● D-glucose, ○, D-xylose, ■, L-arabinose, □, biomass dry weight, ▲, ethanol, Δ, glycerol. Data shown in the figure represent data from one of two independent duplicate experiments for which kinetic parameters differed by less than 5% (Table 3).



**Figure 4** | Growth and extracellular metabolite concentrations in anaerobic batch cultures of **(a. and b.)** synthetic consortia consisting of the evolved glucose-phosphorylation-negative *S. cerevisiae* strains IMS0535 (D-xylose fermenting) and IMS0522 (L-arabinose fermenting (Verhoeven *et al.*, 2018)), together with the D-glucose fermenting laboratory strain CEN.PK113-7D and **c.** pure culture of *S. cerevisiae* IMS0010 (generalist glucose-xylose-arabinose fermenting strain (Wisselink *et al.*, 2009) ). Cultures were growth with different inoculum ratios; **a.** 33% of each strain; **b.** 59% IMS0535, 38.6% IMS0522 and 2.4% CEN.PK113-7D. All cultures were inoculated to a total initial concentration of 0.36 g biomass dry weight L<sup>-1</sup> in bioreactors containing synthetic medium with 20 g L<sup>-1</sup> L-arabinose, 20 g L<sup>-1</sup> D-glucose and 20 g L<sup>-1</sup> D-xylose. List of symbols: ● D-glucose, ○, D-xylose, ■, L-arabinose, □, biomass dry weight, ▲, ethanol, △, glycerol. All three growth experiments were performed in duplicate, data shown are from a single experiment. Kinetic parameters calculated from duplicate cultures differed by less than 5%.



**Figure 5** | **a.** Schematic overview of laboratory evolution experiment with a consortium consisting of the glucose-phosphorylation-negative *S. cerevisiae* strains IMS0535 (D-xylose fermenting) and IMS0522 (L-arabinose fermenting (Verhoeven *et al.*, 2018)) together with the D-glucose fermenting strain CEN.PK113-7D). Laboratory evolution was initiated in anaerobic shake flasks with synthetic medium containing 20 g L<sup>-1</sup> D-glucose, 20 g L<sup>-1</sup> D-xylose and 20 g L<sup>-1</sup> L-arabinose by inoculating with equal amounts of biomass of each of three strains, giving a total initial concentration of 0.36 g biomass dry weight L<sup>-1</sup>. After 3 transfers, strain IMS0522 was re-inoculated and cultivation was continued on SM containing 10 g L<sup>-1</sup> of each sugar. The final shake flask was transferred to anaerobic SBR cultures. **b.** Length of fermentation cycles during the first 21 cycles of SBR cultivation in duplicate (□, reactor 1, ■, reactor 2). **c.** Length of fermentation cycles (○) and off-gas CO<sub>2</sub> profiles for selected cycles during a subsequent series of SBR cycles in the same reactors, in which the sugar composition of the medium contained 20 g L<sup>-1</sup>

D-glucose, 10 g L<sup>-1</sup> D-xylose and 5 g L<sup>-1</sup> L-arabinose. The metabolite concentration and CO<sub>2</sub> measured (*solid grey*) shown in panels **d.** and **e.** correspond to the fifth cycle shown in **b.** and the final cycle of the culture stability experiments shown in **c.**, respectively. List of symbols: ● D-glucose, ○, D-xylose, ■, L-arabinose, □, biomass dry weight, ▲, ethanol, Δ, glycerol. Data shown in the figure corresponds to one reactor of two replicates. Complete off-gas CO<sub>2</sub> profiles for both reactors are shown in Additional File 12.

**Table 1** | *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Reference
CEN.PK 113-7D	<i>MATa MAL2-8c SUC2</i>	(Entian & Kötter, 2007)
IMS0010	<i>MATa ura3-52 leu2-112 loxP-pTPI::( 266, 1)TAL1 gre3::hphMX pUGPTPI-TKL1 pUGPTPI-RPE1 loxP-PTPI::( 40, 1)RKI1 {pRW231, pRW243};</i> strain harboring <i>Piromyces</i> sp. E2 <i>xylA</i> and <i>L. plantarum araA</i> and <i>araD</i> on 2μ -based plasmid pRW231 and <i>XKS1</i> and <i>L. plantarum araB</i> on integration plasmid pRW243; selected for anaerobic growth on L-arabinose, and mixtures of D-xylose, D-glucose and L-arabinose	(Wisselink <i>et al.</i> , 2009)
IMS0522	<i>MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxx1::KLEU2 gal1::cas9- amdS gre3::pTDH3_RPE1 pPGK1_TKL1, pTEF1_TAL1 pPGI1_NQM1 pTPI1_RKI1 pPYK1_TKL2 gal80::(pTPI_araA_tCYC)*9 pPYK-araB-tPGI1 pPGK-araD-tTDH3 hxx2:: PcaraT</i> that has undergone laboratory evolution on mixture of arabinose, glucose and xylose under anaerobic conditions	(Verhoeven <i>et al.</i> , 2018)
IMX604	<i>MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxx1::KLEU2 gal1::cas9- amdS gre3::pTDH3_RPE1 pPGK1_TKL1, pTEF1_TAL1 pPGI1_NQM1 pTPI1_RKI1 pPYK1_TKL2</i>	(Verhoeven <i>et al.</i> , 2018)
IMX659	<i>MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxx1::KLEU2 gal1::cas9- amdS gre3::pTDH3_RPE1 pPGK1_TKL1, pTEF1_TAL1 pPGI1_NQM1 pTPI1_RKI1 pPYK1_TKL2 can1::(pTPI_xylA_tCYC)*9 pTEF1-XKS1</i>	This study
IMX730	<i>MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxx1::KLEU2 gal1::cas9- amdS gre3::pTDH3_RPE1 pPGK1_TKL1, pTEF1_TAL1 pPGI1_NQM1 pTPI1_RKI1 pPYK1_TKL2 can1::(pTPI_xylA_tCYC)*9 pTEF1-XKS1 hxx2:: PcaraT</i>	This study
IMS0524	IMX730 subjected to evolutionary engineering on a mixture of D-xylose, D-glucose and L-arabinose under anaerobic conditions	This study
IMS0533	IMX730 subjected to evolutionary engineering on a mixture of D-xylose, D-glucose and L-arabinose under anaerobic conditions	This study

IMS0535	IMX730 subjected to evolutionary engineering on a mixture of D-xylose, D-glucose and L-arabinose under anaerobic conditions	This study
IMS0537	IMX730 subjected to evolutionary engineering on a mixture of D-xylose, D-glucose and L-arabinose under anaerobic conditions	This study

**Table 2** | Biomass-specific sugar consumption rate ( $q_s$ ), maximum specific growth rate ( $\mu_{\max}$ ) and yields of biomass and ethanol on sugars in anaerobic bioreactor batch cultures of *S. cerevisiae* strains IMS0535 (D-glucose-phosphorylation-negative strain evolved for D-xylose fermentation in presence of L-arabinose and D-glucose), IMS0522 (D-glucose-phosphorylation-negative strain evolved for L-arabinose fermentation in presence of D-xylose and D-glucose), CEN.PK113-7D and IMS0010 (D-glucose, D-xylose and L-arabinose fermenting strain) in synthetic medium containing 20 g L<sup>-1</sup> L-arabinose, 20 g L<sup>-1</sup> D-glucose and 20 g L<sup>-1</sup> D-xylose. Data represent average and mean deviation of measurements on two independent cultures of each strain.

	$q_s$ (g g <sup>-1</sup> h <sup>-1</sup> )			$\mu_{\max}$	Yield (g g <sup>-1</sup> sugar consumed)	
	D-glucose	D-xylose	L-arabinose	(h <sup>-1</sup> )	Biomass	Ethanol
IMS0535	-	0.90 ± 0.13	-	0.09 ± 0.003	0.08 ± 0.002	0.39 ± 0.05
IMS0522	-	-	1.6 ± 0.08	0.12 ± 0.001	0.075 ± 0.002	0.38 ± 0.01
CEN.PK113-7D	2.82 ± 0.05	-	-	0.29 ± 0.01	0.1 ± 0.005	0.40 ± 0.05
IMS0010	1.73 ± 0.07	0.40 ± 0.02	0.57 ± 0.05	0.18 ± 0.004	0.09 ± 0.002	0.41 ± 0.08