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BIOENERGY/BIOFUELS/BIOCHEMICALS

Deletion of *hxk1* gene results in derepression of xylose utilization in *Scheffersomyces stipitis*

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Abstract A major problem in fermenting xylose in lignocellulosic substrates is the presence of glucose and mannose which inhibit xylose utilization. Previous studies showed that catabolite repression in some yeasts is associated with hexokinases and that deletion of one of these gene(s) could result in derepressed mutant strain(s). In this study, the *hxk1* encoding hexokinase 1 in *Scheffersomyces stipitis* was disrupted. The Δ*hxk1* SS6 strain retained the ability to utilize the main hexoses and pentoses commonly found in lignocellulosic hydrolysates as efficiently as the wild-type (WT) strain. SS6 also fermented the dominant sugars to ethanol; however, on xylose, the Δ*hxk1* strain produced more xylitol and less ethanol than the WT. On mixed sugars, as expected the WT utilized glucose ahead of xylose and xylose utilization did not commence until all the glucose was consumed. In contrast, the Δ*hxk1* mutant showed derepression in that it started to utilize xylose even when considerable glucose (about 1.72 %, w/v) remained in the medium. Similarly, mannose did not repress xylose utilization by the Δ*hxk1* mutant and xylose and mannose were simultaneously utilized. The results are of interest in efforts to engineer yeast strains capable of efficiently utilizing glucose and xylose simultaneously for lignocellulosic biomass conversion.

Keywords Glucose repression · Hexokinase · Lignocellulose · Pentose-fermenting · Yeast

Introduction

On hydrolysis, lignocellulosic substrates yield a mixture of hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose). Of these, glucose is the most abundant, followed by xylose, while the other sugars are found in lower concentrations. To improve the process economics, potential fermenting microorganisms must be able to convert all the hexoses and pentoses efficiently to the desired compounds. Native pentose-fermenting yeasts such as *S. stipitis* and *Pachysolen tannophilus* can ferment xylose to ethanol but they do so less efficiently than fermenting glucose. Moreover, in the presence of mixed sugars, they ferment glucose first, leaving xylose incompletely fermented [5, 10, 16]. This is due in part to glucose repression of the induction of xylose reductase and xylitol dehydrogenase, two main enzymes involved in xylose metabolism in native pentose-fermenting yeasts [5, 14]. Poor and inefficient pentose fermentation in the presence of hexoses is recognized as one of the central problems that must be addressed to improve lignocellulosic biomass conversion [8].

It has been known for some time that in *Saccharomyces cerevisiae*, one of the hexokinases is involved in catabolite repression [21]. For example, Entian and Frohlich [6] reported that *hex1*^{r} mutants of *S. cerevisiae* with defect in hexokinase PII (encoded by *hxk2*) activity showed carbon catabolite derepression. In this case, diploid cells were subjected to mutagenesis by ethyl methanesulfonate (EMS) followed by selection on 2-deoxyglucose (2-DG) and raffinose. The *hex1*^{r} mutants (35 out of 3000 tested mutants) with reduced hexokinase PII activity showed glucose

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derepression for invertase, maltase and malate dehydrogenase activities compared to the wild-type (WT) strain. One side effect of a defect in hexokinase activity was longer generation time when mutants were grown on glucose.

In the pentose-fermenting yeast *P. tannophilus*, the *hxk2* gene was shown to be responsible for carbon catabolite repression and its presence is required for repression of xylose reductase and xylitol dehydrogenase [17, 18]. In these studies [17, 18], *P. tannophilus* was subjected to 2-DG or UV treatment, followed by selection on YNB plates supplemented with glucose and xylose. Several mutants were obtained and each had mutation(s) in different genes such as *glu1* (encoding glucokinase 1), *hxk1* (encoding hexokinase 1) and *hxk2* (encoding hexokinase 2) as well as combined mutations in two (*hxk2* and *glu1*) or three (*hxk1, hxk2* and *glu1*) genes. Mutants defective in hexokinase 2 showed glucose derepression and produced the same level of xylose reductase and xylitol dehydrogenase activities on mixed xylose and glucose-grown as xylose-grown cells. However, xylose reductase and xylitol dehydrogenase were fully repressible in mutant defective in glucokinase 1 but with intact hexokinase 2.

In another pentose-fermenting yeast *S. stipitis*, derepressed mutants have been obtained by subjecting cells to EMS mutagenesis followed by selection on either 2-DG alone or 2-DG plus xylose [15]. Several *S. stipitis* mutants were shown to be derepressed for pentose utilization [15]. However, most of them exhibited poor glucose utilization compared to the WT. One mutant (FPL-DX26) stood out as it exhibited partial glucose derepression while retaining the ability to utilize glucose at the same rate as the WT.

Scheffersomyces stipitis possesses 4 hexo- or glucokinase genes [9]. In this study, the *hxk1* gene encoding hexokinase 1 (GenBank Accession number ABN68660.2) in *S. stipitis* was deleted and the ability of one of the Δ*hxk1* mutant (designated SS6) to utilize different individual and mixed sugars was assessed. The results showed that the Δ*hxk1* mutant retained the ability to utilize glucose and xylose individually as well as the WT, but it was derepressed and began to utilize xylose in the presence of appreciable concentration of glucose.

Materials and methods

Strains

Scheffersomyces stipitis WT strain NRRL Y-7124 (NRC 2548) was obtained from the National Research Council Canada (NRCC) Culture Collection (Ottawa, Ontario, Canada). *Escherichia coli* HST04 stellar competent cells were purchased from Clontech Laboratories Inc. (Mountain View, CA, USA).

Culture maintenance and inoculum preparation

Scheffersomyces stipitis WT strain was maintained on YEPD agar plates containing 2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) glucose and 2 % (w/v) agar at 4 °C. *Scheffersomyces stipitis* Δ*hxk1* mutant strains were maintained on YEPD plates supplemented with 5 μg/mL aureobasidin A (AbA) (Clontech) used as the selection marker at 4 °C. The strains were grown in YEPD broth (supplemented with AbA for the mutant strains) at 28 °C and shaken at 180 rpm for 48 h. Equal volume of 50 % (w/v) glycerol was added to cell cultures and kept at −80 °C for long-term storage. For inoculum preparation, a loopful of cells from an isolated colony on YEPD agar plate was transferred to 100 mL of broth containing 0.67 % (w/v) yeast nitrogen base (YNB) without amino acids and ammonium sulfate, supplemented with 0.225 % (w/v) urea and 2 % (w/v) galactose in a 250-mL Erlenmeyer flask. The culture was grown with shaking (180 rpm) at 28 °C for 48 h [2].

Construction of *hxk1* gene deletion cassette

Scheffersomyces stipitis WT was grown in YEPD medium at 28 °C with shaking (180 rpm) for 48 h. The cells were collected by centrifugation (6000×*g* for 5 min) and used for genomic DNA isolation. *Scheffersomyces stipitis* genomic DNA, extracted as previously described [12], was used to obtain the 282- and 254-bp sequences upstream and downstream, respectively, of *hxk1*. Oligonucleotide primers used for PCR amplification are shown in Table 1. Plasmid pAUR123 (Clontech) was used to construct *hxk1* gene deletion cassette as follows: the 282-bp *hxk1* upstream sequence (amplified by *hxk1*-F-U-F1 and *hxk1*-F-U-R1 primers) and 254-bp *hxk1* downstream sequence (amplified by *hxk1*-F-D-F1 and *hxk1*-F-D-R1 primers) were amplified separately by PCR. The primers contained 15 bp homologous sequences on their respective 5′ ends to create the overlapping regions around the AbA cassette of the pAUR123 plasmid. The AbA cassette of the plasmid was amplified using AUR1-C-F-F1 and AUR1-C-F-R1 primers. The ampicillin resistance gene and plasmid origin of replication fragments were amplified using pAUR-F-F3 and pAUR-F-R3 primers. Finally, all four amplified fragments were assembled using In-Fusion Cloning Kit (Clontech) to generate pAUR-hxk1-Disruption plasmid (Supplementary material 1). This plasmid was transformed into *E. coli* HST04 stellar competent cells for maintenance. Putative transformants were screened on LB agar plates supplemented with 50 μg/mL ampicillin. Positive transformants were selected and the correct insertion of *hxk1* disruption cassette into the plasmid was confirmed in one transformant by DNA sequencing using *hxk1*-D-F2 and *hxk1*-U-R2 primers.

Table 1 List of oligonucleotide primers used in this study

Name	Sequence (5′ → 3′)	Gene, fragment size
pAUR-F-F3	GGATCCTCTAGCTCCCTAAC	pAUR123 plasmid backbone, 3355 bp
pAUR-F-R3	CCTCGCGCGTTTCGGTGAT	
hxk1-F-D-F1	CCGAAACGCGCGAGGGCTCTTTCAGTATAGCAGACAT	*hxk1*-down, 254 bp
hxk1-F-D-R1	TTCTCGAAATTCGAGCTGTTCATACAAATGGGTGC	
AUR1-C-F-F1	CTCGAATTTCGAGAAAGTG	AUR1-C, 2320 bp
AUR1-C-F-R1	AACTACTTACACTATAGAGGAAAGAATAACGCAAAA	
hxk1-F-U-F1	ATAGTGTAAGTAGTTGGATAAGG	*hxk1*-up, 282 bp
hxk1-F-U-R1	GGAGCTAGAGGATCCCGACCATAATAACAGTATAGC	
hxk1-D-F2	CTCTTTCAGTATAGCAGACAT	*hxk1*-down/up, 2858 bp
hxk1-U-R2	CGACCATAATAACAGTATAGC	
26S-F1	GGATTGCCTTAGTAACGG	26S rRNA, 1063 bp
26S-R1	CATCGCCAGTTCTGCTTAC	

The homologous sequences to make the overlaps in the proximal regions are underlined

Deletion of *hxk1* and selection of Δ*hxk1* mutants

The pAUR-hxk1-Disruption plasmid was extracted from *E. coli* HST04 cells using QIAprep Spin Miniprep Kit (Qiagen Inc., Montréal, QC, Canada) and linearized by *Xba*I (New England Biolabs Inc., Whitby, ON, Canada) for use in transformation. Competent *S. stipitis* cells were prepared according to the protocol for transformation of *Pichia pastoris* [13]. Briefly, the culture was grown in 25 mL YEPD medium in a 125-mL Erlenmeyer flask at 28 °C and shaken at 180 rpm for 48 h. The cells were used to inoculate 200 mL fresh YEPD medium (initial OD_{600} was 0.1–0.2) in a 1-L Erlenmeyer flask and incubated at 28 °C with shaking (180 rpm) until OD_{600} reached approximately 1.5. The cells were centrifuged at 1500×*g* for 10 min at 4 °C and washed twice with sterile cold water. The cells were washed once with cold 1 M sorbitol and then resuspended in 1 mL of cold 1 M sorbitol followed by transformation using Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories, Mississauga, ON, Canada). Transformants were selected on agar plates containing YNB without amino acids supplemented with 1 M sorbitol, 2 % (w/v) galactose and 5 μg/mL AbA selection marker. Galactose was used as the carbon source, as we were uncertain if putative hexokinase mutants may exhibit slow growth on glucose and hence would not be selected on a glucose-containing medium. Twelve AbA-resistant isolates were randomly selected and tested individually for the correct integration of pAUR-hxk1-Disruption cassette at the *hxk1* locus by PCR using primer pairs that spanned parts of the 5′ and 3′ UTR of the *hxk1* gene (*hxk1*-D-F2 and *hxk1*-U-R2 primers) as well as primers specific for the AbA resistance gene (AUR1-C-F-F1 and *hxk1*-U-R2 primers, Table 1). The PCR products obtained were further confirmed by DNA sequencing and three mutants (designated SS2, SS6 and SS9) with the correct insertion of the *hxk1* gene deletion cassette were obtained. In subsequent sugar utilization study, all three transformants exhibited similar trends. Thus, only the results for SS6 are reported below. SS6 and the WT were subjected to partial 26S rRNA gene (GenBank Accession number JQ689044.1) sequencing analysis using primers 26S-F1 and 26S-R1 (Table 1) that targeted nucleotides 11-1074 of the 3256-bp 26S rRNA gene to confirm their identity.

Sugar utilization and fermentation assessment of the Δ*hxk1* mutant SS6

The Δ*hxk1* strain SS6 was tested for its ability to utilize individual sugars commonly found in lignocellulosic hydrolysates. The strain was also tested for its ability to utilize combinations of mixed sugars (xylose + glucose, glucose + galactose, xylose + galactose and xylose + mannose) using high initial cell density inocula, which were prepared as described [3]. Briefly, 100 mL of 48 h grown inoculum culture was collected and centrifuged at 6000×*g* for 10 min. The initial OD values for mutant SS6 and WT strains were adjusted before starting the fermentation to ensure similar initial cell biomass (OD_{600} around 8.0 ± 1.0) were used in fermentation. After removing the supernatant, the cell pellet was washed twice with sterile water and suspended in 100 mL of 0.67 % (w/v) YNB without amino acids and ammonium sulfate, supplemented with 0.225 % (w/v) urea and 4 % (w/v) of respective carbon sources in a 250-mL flask. The flasks were incubated at 28 °C and shaken at 180 rpm [3]. Culture samples were withdrawn periodically to monitor sugar utilization as well as ethanol and xylitol production.

Analytical methods

Culture samples were centrifuged at 17,000×*g* for 10 min and the supernatant was stored at −20 °C before analysis. Ethanol, xylitol and residual sugar concentrations in the supernatant were measured by HPLC using an Aminex HPX-87H column (Bio-Rad) as described [4] using either isopropanol (for ethanol) or glycerol (for sugars and xylitol) as the internal standard. The mobile phase was 5 mM H_2SO_4, and the column was eluted at a flow rate of 0.6 mL/min at 40 °C. An Aminex HPX-87P column (Bio-Rad) was used for separation of galactose from xylose when a galactose–xylose mixture was used. For these samples, butanol served as the internal standard for ethanol analysis. The mobile phase was H_2O, and the column was eluted at a flow rate of 0.6 mL/min at 60 °C.

All liquid culture experiments were conducted at least three times using independently grown inocula. Since the sampling periods differed for each replicate experiment, figures presented show representative trends from one independent experiment.

Results and discussion

Scheffersomyces stipitis is subject to glucose repression. In potential lignocellulosic hydrolysates, xylose utilization is repressed by glucose and mannose [5]. To enable efficient glucose-xylose co-utilization, it is desirable to obtain glucose-derepressed yeast mutants. Previous reports suggested that one of the hexokinases in *S. cerevisiae* [6] and *P. tannophilus* [18] is involved in catabolite repression. If the same scenario exists in *S. stipitis*, then disruption of the hexokinase associated with catabolite repression may yield a glucose derepressed strain. To test this possibility, we disrupted the *hxk1* gene encoding hexokinase 1 in *S. stipitis* and assessed the ability of the Δ*hxk1* mutant to co-utilize glucose and xylose.

Deletion of *hxk1* gene in *S. stipitis* NRRL Y-7124

A pAUR-hxk1-Disruption cassette was constructed for targeted gene deletion. It was integrated into the *S. stipitis* genomic DNA by homologous recombination. Putative transformants were selected on defined medium agar plates containing 5 µg/mL AbA. About 100 colonies were obtained on each plate after 3–4 days of incubation and 12 colonies were randomly selected for further testing. Successful deletion of the *hxk1* gene in three (designated SS2, SS6 and SS9) of these mutants was confirmed by PCR using primers that spanned parts of the 5′-UTR and 3′-UTR of the *hxk1* gene. This was shown by a 2858-bp band in the mutants instead of 2122-bp band for the WT strain. The result for one of the mutants (SS6) is shown in Fig. 1a. Additionally, successful integration of the deletion cassette into the WT genome was confirmed using primers specific for the AbA resistance gene and *hxk1* downstream homologous sequence. The mutant strain carrying the deletion cassette yielded a 2600-bp band while no band was obtained for the WT strain (Fig. 1b). For further confirmation, the partial sequence of the 26S rRNA gene (about 1060 bp) in SS6 and the WT was amplified separately by PCR followed by sequencing analysis. Both strains were identified as *S. stipitis* with 100 % sequence identity to that in GenBank (Accession number JQ689044.1).

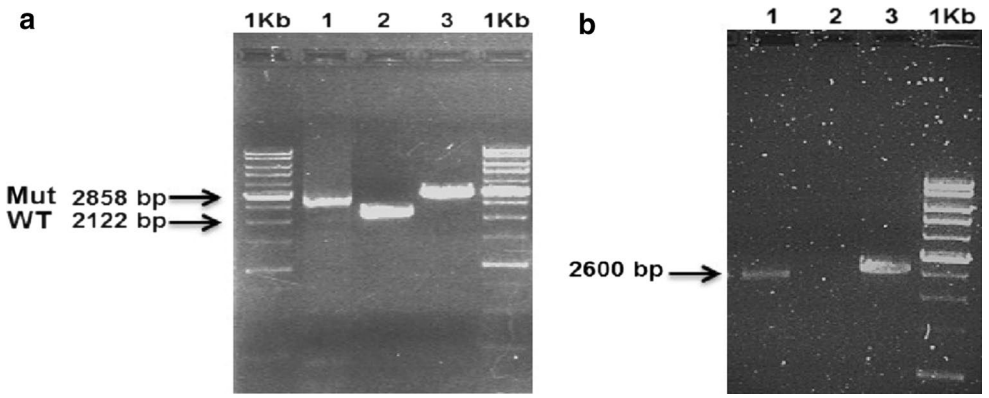

Fig. 1 PCR verification of *hxk1* gene deletion in the Δ*hxk1* SS6 mutant. **a** PCR products obtained using primers specific for *hxk1* upstream and downstream homologous sequences showing a 2858-bp band (*lane 1*) in the SS6 mutant instead of 2122 bp band for the WT (*lane 2*); the pAUR-hxk1-Disruption plasmid was used as the template in a positive PCR control reaction (*lane 3*). **b** PCR products using primers for AbA antibiotic resistance gene and *hxk1* downstream homologous sequence showing a 2600-bp band in the SS6 mutant (*lane 1*) and lack of amplification by the WT (*lane 2*); the pAUR-hxk1-Disruption plasmid was used as the template in a positive PCR control reaction (*lane 3*)

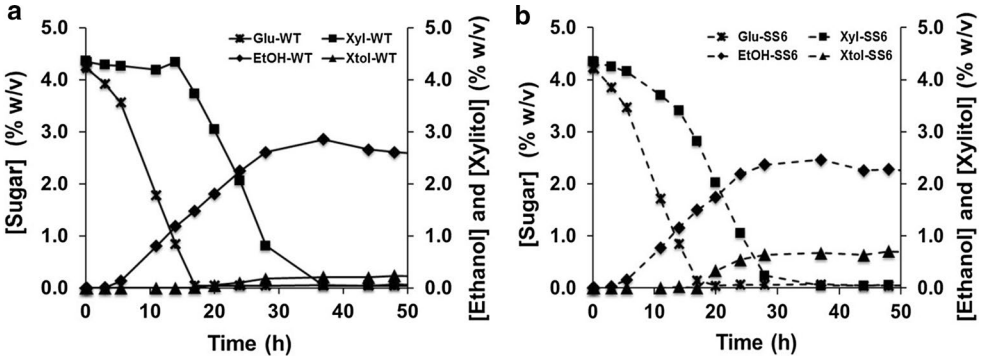

Fig. 2 Fermentation of a mixture of glucose and xylose using high initial cell density inocula. **a** *S. stipitis* WT; and **b** Δ*hxk1* SS6 mutant

Utilization of a mixture of glucose and xylose by SS6

The key goal of this study was to test the hypothesis that knocking out the *hxk1* gene would alleviate glucose repression, thereby allowing for glucose–xylose co-utilization by *S. stipitis*. Thus, the ability of the Δ*hxk1* SS6 strain to utilize and ferment a mixture of glucose and xylose was first assessed in defined media using high initial cell density inocula. As expected, the WT strain consumed glucose before xylose (Fig. 2a). Glucose (4 %, w/v) was completely utilized in about 17 h. Xylose utilization by the WT only began after all the glucose was depleted. SS6 consumed glucose as rapidly as the WT and glucose was completely consumed around 17 h. The *S. stipitis* Δ*hxk1* strain possesses 3 other hexo- or gluco-kinases (encoded by *nag5*, *glk1* and *glk2*) [9] which likely allowed glucose to be rapidly phosphorylated and consumed.

Similar results were observed in *S. cerevisiae* when the *hxk2* gene encoding hexokinase 2 was disrupted in that the mutant utilized glucose at the same rate as the WT in a glucose-galactose mixture [1]. In contrast, in *P. tannophilus*, disruption of one of the hexokinases (*hxk2*) led to reduced rate of glucose consumption [17, 18]. In these *P. tannophilus* studies, mutant P510-5A with defect in glucokinase 1 (encoded by *glu1*) utilized glucose as well as the WT. Also, xylose reductase and xylitol dehydrogenase were fully repressible by glucose. However, mutant P509-1B, with mutations in *hxk2* and *glu1*, took 70 h to completely consume 2 % (w/v) glucose, in contrast to 16 h by the WT. The mutant consumed 2 % (w/v) xylose at the same rate as the WT (about 70 h). Furthermore, P509-1B was derepressed for xylose reductase and xylitol dehydrogenase. The same results were observed with mutant P509-3C which carried only the *hxk2* mutation. These results confirmed the central role of *hxk2* in catabolite repression in *P. tannophilus* [17, 18].

In *Kluyveromyces lactis*, mutants with defect in *Rag5* (encoding hexokinase) exhibited slower growth rate on glucose [7, 19]. Furthermore, a double mutant with defect in two genes (*dgr148* and *dgr239*) which regulate hexokinase activity was derepressed for invertase, maltase and β-galactosidase [7, 19].

Sreenath and Jeffries [15] subjected *S. stipitis* CBS 6054 (NRRL Y-11545) cells to EMS mutagenesis followed by selection on 2-DG or 2-DG plus xylose. Selection on 2-DG plus xylose led to the isolation of one mutant (FPL-DX26) which exhibited partial derepression in that in a glucose-xylose mixture, xylose consumption began when some glucose (about 1 %, w/v) remained in the medium [15]. In all the aforementioned studies, deletion of one of the hexokinases resulted in glucose derepression. Also, some, but not all, of the hexokinase mutants showed slower glucose utilization.

The rapid consumption of glucose retained in the *S. stipitis* Δ*hxk1* SS6 mutant is a desired trait for lignocellulosic biomass conversion. In our study, glucose was utilized before xylose by SS6. However, xylose utilization began before all the glucose was completely consumed. This was discernible at 5.5 and especially 11 h when considerable glucose (about 1.72 %, w/v) remained in the medium (Fig. 2b). Xylose was completely consumed by both WT and SS6 strains in about 37 h. The maximum ethanol concentrations produced by the WT and SS6 strains were 2.85 and 2.46 % (w/v), respectively, at 37 h. Maximum xylitol concentrations of 0.24 and 0.7 % (w/v) were produced at 48 h by the WT and SS6 strains, respectively. The Δ*hxk1* mutant clearly displayed glucose derepression and one side effect was increased production of xylitol at the expense of ethanol. The reason for higher xylitol production by SS6 compared to the WT is not known.

The Δ*hxk1* SS6 mutant obtained in this study bears some resemblance to the FPL-DX26 mutant described by Sreenath and Jeffries [15]. FPL-DX26 utilized glucose in a mixed glucose and xylose (3 %, w/v each) fermentation as fast as the WT strain and glucose was utilized within 40 h. However, a key difference is that SS6 produced more xylitol but less ethanol than the WT. In the study of Sreenath and Jeffries [15], it was not indicated if FPL-DX26 produced more xylitol than the WT. However, given that it produced more ethanol than the WT, it is unlikely to

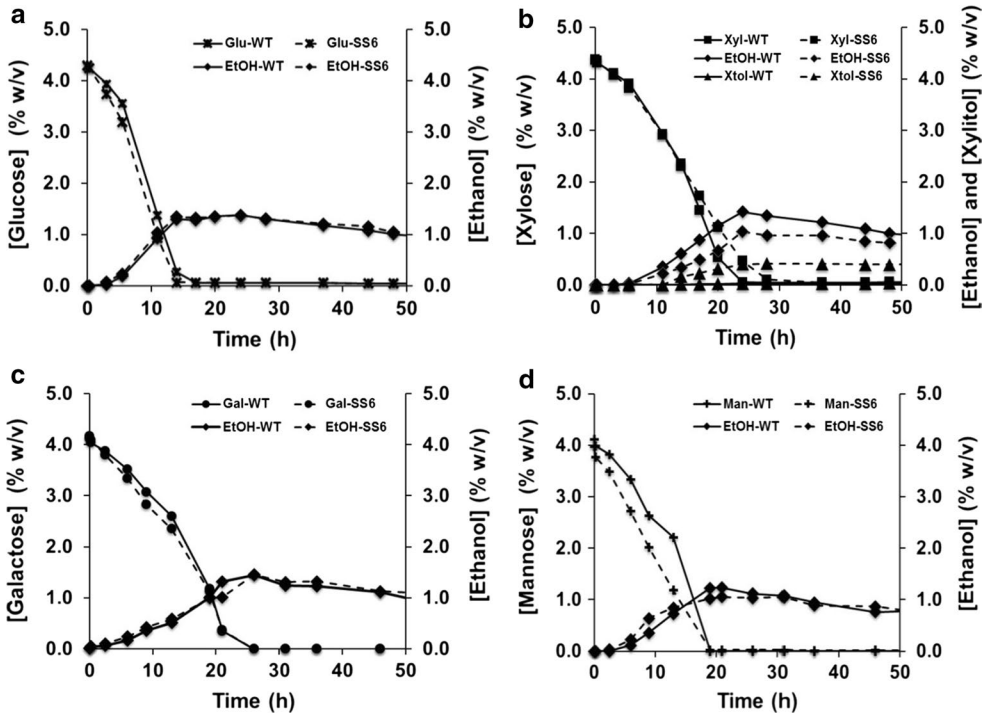

Fig. 3 Fermentation of individual sugars by *S. stipitis* WT and Δ*hxk1* SS6 strains using high initial cell density inocula. **a** 4 % (w/v) glucose; **b** 4 % (w/v) xylose; **c** 4 % (w/v) galactose; and **d** 4 % (w/v) mannose

be producing more xylitol as seen in SS6. FPL-DX26 was selected on the basis of 2-DG resistance. Although the specific mutation(s) in this strain was not determined, 2-DG is thought to target one of the hexokinase genes [11, 20]. Thus, FPL-DX26 likely had defect in one of the 4 hexo- or gluco-kinases in *S. stipitis*, but the defect differs from the *hxk1* specifically deleted in SS6. One word of caution about this conclusion is that different *S. stipitis* strains were used in the study by Sreenath and Jeffries [15] and our current study. It is not known if deletion of the same gene in different *S. stipitis* strain may give rise to different fermentation phenotype.

The inocula for the experiment shown in Fig. 2 were grown in galactose which would not induce or repress xylose reductase or xylitol dehydrogenase activity in *S. stipitis* [5]. In a separate experiment, the inocula were grown in xylose to induce xylose reductase and xylitol dehydrogenase activity. The results on mixed glucose–xylose utilization by high cell density inocula of the xylose-grown inocula (data not shown) were similar to those obtained using galactose-grown inocula presented in Fig. 2. Thus, high initial cell density inocula grown on galactose were used for all subsequent sugar utilization and fermentation experiments.

Single sugar utilization by SS6

The ability of the Δ*hxk1* SS6 and WT strains to utilize and ferment individual sugars (glucose, xylose, galactose or mannose) commonly found in lignocellulosic hydrolysates was assessed in chemically defined media using high initial cell density inocula (Fig. 3a–d). Arabinose utilization was not assessed as *S. stipitis* was known to utilize arabinose slowly with no ethanol production [3].

The utilization patterns of individual sugars by SS6 were similar to those of the WT strain. Glucose at 4 % (w/v) was completely utilized by WT and SS6 within 14 h (Fig. 3a). The maximum ethanol concentration produced by both WT and SS6 was about the same at 1.38 % (w/v) after 24 h. Similar results were reported by Bajwa et al. [3] using high initial cell density of the *S. stipitis* WT. Xylose (4 %, w/v) was utilized and fermented to ethanol by both WT and SS6 at a slower rate than glucose (Fig. 3b). SS6 consumed all the xylose within the first 26 h which was slightly slower compared to WT (24 h). Maximum ethanol concentrations of 1.42 % and 1.04 % (w/v) were produced by the WT and SS6, respectively, at 24 h. Xylitol production by the WT was negligible (about 0.02 %, w/v), while the mutant produced a peak xylitol concentration of 0.4 % (w/v) at 24 h (Fig. 3b). Thus, it appears that considerable amount of xylose was channeled to xylitol production and this led to a decrease in ethanol production by SS6, as seen also during mixed glucose-xylose fermentation (Fig. 2b). Galactose at 4 % (w/v) was completely utilized by both strains within 26 h (Fig. 3c) and about the same level of peak ethanol concentration (1.4 %, w/v) was produced at 26 h. Mannose (4 %, w/v) was completely utilized after 19 h and the peak ethanol concentrations for the mutant and WT ranged from 1.02 to 1.23 % (w/v), respectively (Fig. 3d).

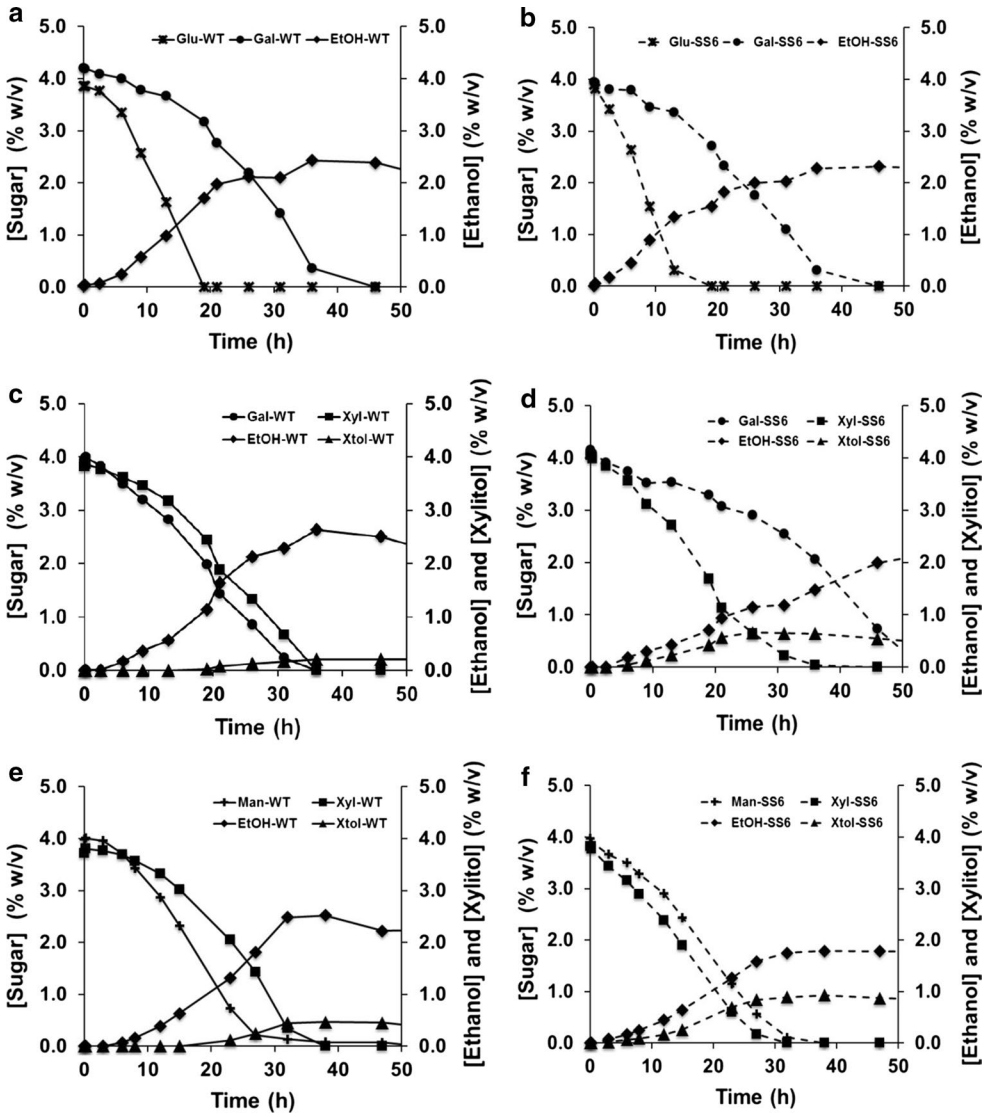

Fig. 4 Mixed sugar fermentation by *S. stipitis* WT and Δ*hxk1* SS6 strains using high initial cell density inocula. Fermentation of **a** glucose and galactose by WT and **b** SS6; **c** xylose and galactose by WT and **d** SS6; and **e** xylose and mannose by WT and **f** SS6

Utilization and fermentation of other mixed sugar combinations by SS6

The Δ*hxk1* SS6 strain was also tested for its ability to utilize combinations of other mixed sugars using high initial cell density inocula. Figure 4a, b shows sugar utilization and fermentation patterns of 4 % (w/v) each of glucose and galactose by the WT and SS6 strains, respectively. Glucose was completely utilized in 19 h while galactose was utilized in 46 h by both strains. Thus, glucose repressed the utilization of galactose in both strains, and galactose utilization began when some (about 1 %, w/v) glucose remained in the medium. The mutant and WT produced similar peak ethanol concentrations (2.3 and 2.4 %, w/v, respectively) at 36 h.

In a galactose–xylose mixture, the WT utilized galactose and xylose simultaneously, and both sugars were completely consumed in about 36 h (Fig. 4c). In contrast, while SS6 utilized xylose and galactose simultaneously, xylose was consumed faster than galactose (Fig. 4d). The maximum ethanol concentrations produced by the WT and SS6 were 2.6 and 2.1 % (w/v) at 36 and 52 h, respectively, while the maximum xylitol concentrations of 0.2 and 0.66 % (w/v) were produced at 36 and 26 h by the WT and SS6, respectively.

Xylose utilization is also repressed by mannose in *S. stipitis* WT [5]. Thus, the ability of SS6 and WT to utilize and ferment a mannose–xylose mixture (each at 4 %, w/v) was tested. As shown in Fig. 4e, the WT utilized mannose ahead of xylose. The onset of xylose utilization started at about 12 h when mannose concentration was about 2.87 % (w/v) which indicated incomplete repression of xylose utilization by mannose in the WT. In contrast, mannose did not repress xylose utilization by the Δ*hxk1* SS6 mutant and xylose and mannose were simultaneously utilized (Fig. 4f). Mannose and xylose were completely utilized by both the

WT and SS6 in about 38 h. The maximum ethanol concentrations produced by the WT and SS6 strains were 2.52 and 1.79 % (w/v) at 38 h, respectively. The maximum xylitol concentrations of 0.47 and 0.93 % were produced by the WT and SS6 strains, respectively, at 38 h.

In summary, our results demonstrate that deletion of the *hxk1* gene in *S. stipitis* resulted in a derepressed mutant strain which retained the ability to efficiently utilize the main sugars in lignocellulosic hydrolysates and ferment them to ethanol. In addition, the mutant was capable of utilizing and fermenting xylose in the presence of considerable amount of glucose or mannose. Efficient co-utilization of glucose and xylose is one of the main targets in efforts to develop robust yeast strains able to overcome the poor utilization of pentoses in mixed sugar substrates such as those found in industrially relevant lignocellulosic hydrolysates.

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