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Assessing the potential of wild yeasts for bioethanol production

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Abstract

Bioethanol fermentations expose yeasts to a new, complex and challenging fermentation medium with specific inhibitors and sugar mixtures depending on the type of carbon source. It is therefore suggested that the natural diversity of yeasts should be further exploited in order to find yeasts with good ethanol yield in stressed fermentation media. In this study, we screened more than 50 yeast isolates of which we selected 5 isolates with promising features. The species *Candida bombi*, *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* showed better osmo- and hydroxymethylfurfural tolerance than *Saccharomyces cerevisiae*. However, *S. cerevisiae* isolates had the highest ethanol yield in fermentation experiments mimicking high gravity fermentations (25% glucose) and artificial lignocellulose hydrolysates (with a myriad of inhibitors). Interestingly, among two tested *S. cerevisiae* strains, a wild strain isolated from an oak tree performed better than Ethanol Red, a *S. cerevisiae* strain which is currently commonly used in industrial bioethanol fermentations. Additionally, a *W. anomalus* strain isolated from sugar beet thick juice was found to have a comparable ethanol yield, but needed longer fermentation time. Other non-*Saccharomyces* yeasts yielded lower ethanol amounts.

Introduction

In order to shift away from a petroleum-based energy source the European Union wants to establish the use of waste organic biomass as a renewable energy source. Currently used (so-called "first generation") organic biomass sources are regular feedstocks with high sugar content (such as wheat, maize, sugar cane or beet) [34]. Whereas these sources might be suitable for high-end, but low quantity products, their role in bulk production of chemicals such as bioethanol is debated as they are competitive with food production. Indeed, it can cause an increase of the food price due to direct competition with the foodstock or through competition with agricultural land [7, 15]. Lignocellulosic biomass has therefore received increasing attention as an alternative for food energy crops. It is a renewable source originating from plant material, widely available and relatively inexpensive and can be non-competitive with food production [14].

The improved use of these types of biomass for bioethanol production yields some hurdles to be tackled. Very high gravity fermentations using first generation biomass [3] imply a high osmotic stress due to high sugar concentrations and high ethanol stress due to ethanol production during fermentation. Main added-value characteristics for bioethanol production strains are therefore high osmo- and ethanol tolerance. Lignocellulosic biomass is a more diverse and complex biomass compared to first generation biomass. It mainly consists of the sugar polymers cellulose and hemicellulose and the phenolic polymer lignin [18, 27, 29]. The cellulose fraction contains glucose monomers, while hemicellulose consists of a variety of C6 (mainly glucose) and C5 sugars (mainly xylose). These sugars are bound into the plant matrix and therefore need to be made available for fermentation by, for example, hydrolysis using acid or base at high temperature [22, 29, 37]. The liberated sugar concentration depends on the treatment type and intensity with high intensity treatments yielding higher sugar concentrations [37]. Typical total sugar concentrations vary between 100 to 200 g l^{-1} with glucose concentrations about double of xylose concentrations [37]. While C6 sugars are readily fermented to ethanol by conventional yeasts such as *Saccharomyces cerevisiae*, *S. cerevisiae* is unable to utilize xylose [4]. The fermentation of xylose, however, contributes to an economically viable second generation bioethanol. Xylose can be fermented by some other yeasts, bacteria or fungi although by-product formation or slow xylose conversion may limit their economic application for ethanol production [32, 34]. Recent research showed the potential of genetically engineering *S. cerevisiae* for the fermentation of xylose [10].

However, these intense treatments to release sugars also result in the formation of several undesired compounds in concentrations which may reduce the fermentation efficiency [9, 14, 18, 20, 37]. Most common inhibitors are weak acids such as levulinic acid, formic acid, acetic acid, furans such as 5-hydroxymethylfurfural (HMF) and furfural and phenolic compounds resulting from the lignin fraction such as vanillin [14, 21, 37]. Typical concentrations of these compounds in hydrolysates are very variable as these are dependent on the biomass source, as well as the treatment procedure and intensity [37].

Due to the experience using *S. cerevisiae* in other industrial fermentations and the large amount of knowledge that has been gathered this species is also the first choice for bioethanol production [5]. It is especially useful in first generation bioethanol production due to its high ethanol yield and ethanol tolerance [38]. However, as outlined above, the fermentation environment in second generation bioethanol production differs greatly from any previous fermentation. In addition, due to large diversity of biomass sources and hydrolysis techniques and the concomitant diversity of the resulting hydrolysate mixture composition, it is required to align the hydrolysate with a yeast with the ideal characteristics. Therefore the natural diversity of yeasts should be further exploited in order to find yeasts with good ethanol yield in a fermentation medium containing high concentrations of glucose, containing a mixture of sugars such as glucose and xylose and/or a myriad of inhibitors. Surprisingly, so far only little is known on the relative ethanol yield of especially non-*Saccharomyces* yeasts under these conditions compared to *S. cerevisiae*.

In this study we examined several yeast species belonging to *Candida, Starmerella, Metchnikowia, Pichia, Hanseniaspora*, *Torulaspora, Wickerhamomyces* and *Citeromyces* which have been isolated from soil or sugar-rich habitats (floral nectar or sugar beet thick juice) for tolerance to inhibitors. Floral nectar and sugar beet thick juice are known for their low water activity (a_w less than 0.9) due to high sugar concentrations and typically contain a limited number of specialized, xerotolerant yeasts [26]. In addition, nectar is also known for its diverse sugar composition [31]. In contrast to these sugar-rich habitats, soil contains a wide variety of different microorganisms. First, a large culture collection was screened for tolerance to osmotic stress, ethanol and HMF. Subsequently, tolerance to weak acids and furfural was examined on a selection of tolerant strains. Finally, 1.1 l fermentation experiments with 25% glucose and experiments mimicking lignocellulosic hydrolysates were performed using the most promising strains and ethanol yield was recorded.

Materials and Methods

Culture collection

A culture collection was constructed consisting of 56 yeast strains previously isolated from soil, plant nectar and sugar beet thick juice, i.e. an intermediate product from beet sugar production (Table 1) [17, 19]. Except for soil, these habitats are known to be low in microbial diversity due to high osmotic stress. In total, 39 nectar isolates were included in this study, representing isolates from *Candida bombi* (14 isolates), *Hanseniaspora uvarum* (5), *Metchnikowia reukauffii* (11) and *Starmerella bombicola* (9). *C. bombi* and *M. reukauffii* were isolated from nectar of the same flower [17]. Further, the collection consisted of 12 isolates from beet sugar thick juice, representing isolates of *Citeromyces matritensis* (5), *Torulaspora delbrueckii* (3) and *Wickerhamomyces anomalus* (4) . From soil one *Pichia kudriavzevii* isolate, one *T. delbrueckii* isolate and three *Metchnikowia pulcherrima* were included. Identifications were based on sequence analysis of the D1/D2 region of the large ribosomal subunit after PCR on the DNA extract [25] using primers NL1 and NL4 [24]. PCR conditions were as follows: 2 min at 94 °C, 35 cycli of 45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, followed by 10 min at 72 °C using Titanium Taq ((Clontech Laboratories, USA). Identification was performed by BLAST analysis in GenBank. To evaluate the phenotypic profile of these isolates in bioethanol production, a commercial *S. cerevisiae* strain currently used in bioethanol fermentation (Ethanol Red) and a 'wild' strain from oak (SCOak50) were used as a reference. All strains were stored in Yeast extract Peptone Dextrose (YPD) broth containing 25% glycerol at -80 °C.

Phenotypic profiling

First, the whole culture collection was screened on solid agar plates for a number of desirable traits, including osmotolerance (glucose 40-70 % w/v), ethanol tolerance (5-15 % v/v) and HMF tolerance (2-7 g l^{-1}) as described by Mukherjee et al. [28]. Briefly, a basic growth medium (control medium) was prepared using bacto peptone 2% w/v (BD), yeast extract 1% w/v (LabM), glucose 2% w/v and agar 1.5% w/v (Invitrogen) . Further, test media were prepared containing the same basic composition as the control medium, but supplemented with the test compounds. Agar plates were prepared using

Singer PlusPlates designed for use with the Singer ROTOR HDA robot (Singer Instruments, UK). Next, the 96-well plate containing the strains stored at -80 °C was thawed, spotted using the HDA robot on control medium containing 2% glucose and incubated at 30 °C for 2 days. Next, a 96-well plate containing 150 µl of liquid control medium in each well was inoculated with the strains using the robot and incubated overnight at 30 °C at 900 rpm for preculturing. In case of screening for ethanol tolerance, strains were precultured for 48 h in liquid control medium with 2% v/v ethanol for preconditioning. Then, optical density at 600nm (OD_{600}) was measured using a microplate reader (Molecular Devices, USA) and the cell density was manually adjusted to OD_{600}≈0.2. This plate was used as the source plate for spotting the test plates with the HDA rotor. After 5 days of incubation at 30 °C all test plates were scanned using a high definition scanner (Seiko Epson, Japan) and processed using ImageJ [1], combined with the ScreenMill software [11] especially developed to quantify the colony size of each isolate on the plates. Relative growth was calculated as the growth at a certain test condition relative to the growth on the control medium. Growth under a test condition is only considered when the relative growth exceeds 5% of the growth on the control medium.

In a second phase, the most promising strains (i.e. strains with good ethanol and HMF tolerance) were further evaluated for their growth in 2% w/v xylose liquid yeast extract bactopeptone (YP) medium. Growth in 2% xylose medium was evaluated by measuring the OD after 24 h of incubation at 30 °C in comparison with their growth in liquid medium containing 2% glucose. Additionally, tolerance to weak acids and furfural, both important inhibitors related to lignocellulosic hydrolysates was assessed. The assay was conducted in liquid medium using the OmniLog incubator/reader (Biolog, USA) which records color development due to metabolic activity. The assay was adapted from the general protocol as supplied by the manufacturer (Biolog, USA). The assay was performed in a 96-well plate in a total volume of 120 µl growth medium, consisting of yeast extract (10g l^{-1}), bacto peptone (20 g l^{-1}), 6% w/v glucose and Biolog dye D (0.5x tetrazolium redox dye) [13, 35]. Inhibitors were added at a concentration ranging from 10 to 40 mM for acetic acid (0.625-2.5 g l^{-1}) and from 5 to 20 mM for furfural (0.48-1.92 g l^{-1}), vanillin (0.76-3.04), formic acid (0.13-0.5 g l^{-1}) and levulinic acid (0.58-2.3 g l^{-1}). Prior to inoculation isolates were overnight pregrown on solid YPD agar and suspended in sterile

demineralized water to an OD_{600} of 0.2. Next, 125 µl was added to 3 ml sterile water of which then 90 µl was used as inoculum. The plates were incubated in the OmniLog instrument at 30 °C and the color development (due to the production of formazan from tetrazolium) at 36 h was recorded. Tolerance values were expressed relative to the growth without any inhibitors.

Fermentation potential of selected isolates in high gravity and lignocellulosic medium

Finally, these most promising isolates (one isolate from *M. pulcherrima*, *P. kudriavzevii*, *T. delbrueckii*, *W. anomalus*) were subjected to small scale fermentations (1.1 l) to estimate their ability to produce ethanol under high gravity (25% glucose) and lignocellulosic hydrolysate stress. As a reference the *S. cerevisiae* strains Ethanol Red and SCoak50 were included. Both strains had previously been shown to have an excellent tolerance profile (Table 1) [28]. Isolates were precultured overnight in 5 ml YP medium containing 2% glucose, followed by inoculation in 300 ml YP medium containing 10% glucose and incubation at 30 °C, 150 rpm for 2 days until stationary phase. Next, OD_{600} was measured and a volume of it was harvested by centrifugation (5 minutes at 3000 rpm) that represented OD_{600}≈2.1 and OD_{600}≈1.5 in 1.1 l high gravity and lignocellulosic hydrolysate stress fermentation medium, respectively. The latter fermentation medium consisted of 4.8% w/v glucose, 6.2% w/v xylose, 0.5% w/v mannose, 0.5% w/v galactose and 0.5% w/v arabinose and an inhibitor concentration of 1.24 g l^{-1} HMF, 0.4 g l^{-1} furfural, 2.3 g l^{-1} acetic acid, 0.44 g l^{-1} formic acid, 0.87 g l^{-1} levulinic acid and 0.04 g l^{-1} vanillin as described by Koppram et al. [21]. Concentrations are within the range tested in the liquid assay test. Batch fermentations were carried out using Eppendorf BioFlo reactors at a pH of 4.5, 30 °C, 300 rpm and saturated with air at the start of the fermentation. After the start of the fermentation dissolved oxygen was not further controlled and dropped quickly due to fermentation activity. During the fermentation OD_{600} was measured frequently and 1 ml samples of the fermentation medium were taken, centrifuged and concentrations of ethanol, glucose and xylose in the supernatant were quantified using high performance liquid chromatography (HPLC) (Waters® isocratic Breeze™ HPLC, ion exchange column WAT010290). Column temperature was maintained at 75 °C, and 5 mM H_2SO_4 was used as eluent with a flow rate of 1 ml min^{-1}. A refractive index detector (Waters 2410, Waters, Milford, MA, USA) was used to detect the compounds of interest.

Results

Tolerance differences between yeast species of different genera

In general, the non-*Saccharomyces cerevisiae* isolates were more tolerant to high glucose concentrations (>55%) than the two selected *S. cerevisiae* reference isolates (Table 1). These were previously shown to be among the top 20 most osmotolerant isolates from a collection of 280 *S. cerevisiae* isolates [28]. Among the isolates from nectar, *Candida bombi* isolates showed the highest tolerance to glucose with an average relative growth (relative to the growth on 2% glucose) of 23% at a concentration of 55% glucose, and 24% at both 60 and 70% glucose. None of the nectar isolates from other genera, even when isolated from the same type of plant nectar, managed to grow at this concentration. Second best among the nectar yeasts was *Starmerella bombicola* with 24% relative growth at 55% glucose, 18% at 60% glucose, but none of the isolates did grow at 70% glucose (Table 1). Among the isolates from sugar beet thick juice, all isolates of *Citeromyces matritensis* managed to grow up to 60% glucose (25% relative growth), and three out of five isolates showed minor growth at 70% glucose. In contrast, the maximal glucose concentration at which isolates of *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* isolates could grow was 55% (28% and 29% relative growth, respectively, Table 1). *Pichia kudriavzevii* (soil) and *Hanseniaspora uvarum* (nectar), represented the least glucose-tolerant non-*Saccharomyces* species, and were able to grow up to 48% and 50% glucose, respectively (Table 1). As expected, *S. cerevisiae* strains were most tolerant to ethanol, tolerating 13% (SCOak50) and 14% (Ethanol Red) ethanol. *P. kudriavzevii* isolated from soil was tolerant to 13% ethanol (Table 1). Non-*Saccharomyces cerevisiae* isolates showed considerable lower ethanol tolerance up to only 11% v/v, with *M. reukauffi*, *M. pulcherrima* and *H. uvarum* showing no growth from 7% v/v ethanol onwards. At 10% and 11% ethanol only *W. anomalus* (18% relative growth) and 1 *T. delbrueckii* isolate (5%), all isolated from thick juice, were able to grow (Table 1). *C. bombi* and *P. kudriavzevii* isolates were the most tolerant to HMF as 42 and 39% relative growth was recorded at 7 g l^{-1} HMF, respectively. Other isolates did not manage to grow at this concentration, except for *S. cerevisiae* SCOak50 (Table 1).

Table 1: Average relative growth (relative to the growth on 2% glucose)[a] of different species on agar plates containing increasing concentrations of glucose, ethanol[b] and 5-hydroxymethylfurfural with the standard error in brackets[c]. The number of isolates incorporated in the screening (#) as well as the isolation source are given.

			Glucose (% w/v)							Ethanol (% v/v)					5-hydroxymethylfurfural (g l^{-1})					
Species	**#**	**Source**	**40**	**46**	**48**	**50**	**55**	**60**	**70**	**5**	**7**	**10**	**11**	**12**	**2**	**3**	**4**	**5**	**6**	**7**
Candida bombi	14	Nectar	62 (3)	47 (2)	41 (2)	35 (2)	23 (1)	24 (1)	24 (2)	52 (3)	27 (2)	0	0	0	98 (4)	108 (3)	108 (3)	87 (4)	68 (4)	51 (3)
Hanseniaspora uvarum	5	Nectar	27 (1)	19 (0.4)	11 (2)	8 (1)	0	0	0	18 (3)	0	0	0	0	47 (1)	13 (2)	12 (2)	0	0	0
Metchnikowia reukauffii	11	Nectar	31 (1)	28 (2)	23 (1)	17 (1)	10 (1)	7 (1)*	0	0	0	0	0	0	49 (6)	**17** (8)	**17** (6)	0	0	0
Starmerella bombicola	9	Nectar	58 (1)	50 (2)	43 (1)	35 (1)	24 (1)	18 (1)	0	18 (2)	**11** (2)	0	0	0	79 (6)	66 (10)	62 (9)	25 (8)*	**39** (2)	0
Metchnikowia pulcherrima	2	Soil	75 (1)	46 (2)	46 (3)	36 (5)	37 (2)	19 (4)	9	15 (3)	0	0	0	0	125 (13)	125 (23)	132 (31)	13 (0)	0	0
Pichia kudriavzevii	1	Soil	114	14	9	0	0	0	0	116	120	85	64	42	78	70	71	57	46	39
Citeromyces matritensis	5	Thick juice	76 (3)	43 (4)	67 (4)	51 (5)	36 (4)	25 (3)	12 (2)*	26 (3)*	**19**	0	0	0	12 (2)*	0	0	0	0	0
Torulaspora delbrueckii	4	Thick juice (3)	87 (31)	39 (2)	45 (2)	22 (7)	29 (3)	6 (1)*	0	57 (5)	36 (4)	**19**	**5**	0	45 (16)*	46 (12)*	30 (14)*	**22**	**8**	0
Wickerhamomyces anomalus	4	Thick juice	161 (37)	45 (10)	45 (10)	28 (6)	28 (4)	0	0	80 (10)	59 (7)	32 (4)	18 (4)	0	86 (14)	74 (16)	69 (15)	24 (9)	11 (4)*	0
S. cerevisiae (EtOH Red)	1	Bio-ethanol	42	42	24	15	0	0	0	101	102	85	81	73	138	129	129	33	0	0
S. cerevisiae (SCOak50)	1	oak	39	44	25	17	0	0	0	94	95	79	73	60	86	75	87	86	38	15

[a] Underlined values: 1 isolate did not grow and was not taken into account; * two isolates did not grow; Bold and underlined values: more than 50% of the isolates did not grow

[b] Ethanol tolerance was also screened above 12% ethanol. For 13% ethanol only *P. kudriavzevii* (14% growth) and both *S. cerevisiae* strains (33% on average) could grow. Only *S. cerevisiae* from bioethanol could grow on 14% ethanol (22%)

[c] No standard errors are given in cases when only 1 isolate is involved or when none of the isolates grew

Table 2: Growth on 2% glucose (colony area, mm^2), growth on 2% xylose (%, relative to growth on 2% glucose) and tolerance to glucose, ethanol and HMF (%, relative to growth on 2% glucose without inhibitors) and tolerance to weak acids, furfural and vanillin (%, relative to metabolic activity in 6% glucose medium) of the best performing isolates of 5 genera.

Strain	***Species***	**Isolation source**	**Glucose (% w/v)**					**Xylose (% w/v)**	**Ethanol (% v/v)**			**HMF ($g\ l^{-1}$)**			**Acetic acid ($g\ l^{-1}$)**	**Formic acid ($g\ l^{-1}$)**	**Levulinic acid ($g\ l^{-1}$)**	**Furfural ($g\ l^{-1}$)**	**Vanillin ($g\ l^{-1}$)**
			2	**46**	**55**	**60**	**70**	**2**	**5**	**11**	**12**	**4**	**5**	**7**	**2.5**	**0.5**	**2.3**	**1.44**	**0.76**
Ethanol Red	*Saccharomyces cerevisiae*	Bioethanol	742	42	0	0	0	3.5	101	81	73	129	33	0	94	89	94	6	86
SCOak50	*Saccharomyces cerevisiae*	Oak	707	44	0	0	0	9	94	73	60	87	86	15	106	105	125	9	88
ST1312/239 MP	*Metchnikowia pulcherrima*	Soil	654	43	34	21	9	25	10	0	0	170	0	0	110	83	90	0	30
ST1312/061 PA G2	*Wickerhamomyces anomalus*	Thick juice	1127	25	19	0	0	24	73	22	0	61	30	0	89	94	89	0	99
ST1312/167 TD	*Torulaspora delbrueckii*	Thick juice	1465	39	24	0	0	35	70	5	0	56	22	1	89	94	83	43	53
ST1312/230 PK	*Pichia kudriavzevii*	Soil	1615	14	0	0	0	43	116	64	42	71	57	39	130	114	121	36	55

However, at a more relevant HMF concentration of 4 g l^{-1} 15 isolates did not manage to grow, i.e. all *C. matritensis* isolates, 7 *M. reukauffii* isolates and 1 *C. bombi*, *H. uvarum* and *T. delbrueckii*. All *W. anomalus* (69%), *M. pulcherrima* and *S. bombicola* strains (62% growth) were able to grow (Table 1). *M. reukauffii* isolates showed variable tolerance, with four out of 11 isolates growing at 4 g l^{-1} HMF (17% growth), while the others did only grow up to 2 g l^{-1}. Surprisingly, while the two *M. pulcherrima* isolates from soil showed 132% growth at 4 g l^{-1}, one did not grow at higher concentrations and the other one only 13% at 5 g l^{-1} (Table1).

Growth on xylose and tolerance to weak acids of selected yeast strains

Due to their very low ethanol (less than 10% v/v ethanol) and/or HMF tolerance isolates of *C. bombi*, *H. uvarum*, *S. bombicola, C. matritensis* and *M. reukauffii* were abandoned for further experiments as they have not the appropriate features for bioethanol production. From the species *W. anomalus*, *M. pulcherrima*, *T. delbrueckii* and *P. kudriavzevii* we selected 1 strain with the largest tolerance to glucose, ethanol and HMF (Table 2), and also both reference *S. cerevisiae* isolates were included. Growth in xylose medium was slower for all tested isolates compared to growth in glucose medium. Non *S. cerevisae* isolates grew generally better on xylose than *S. cerevisiae* strains (<5%), especially *W. anomalus* PA G2, *T. delbrueckii* 167TD and *P. kudriavzevii* 230PK with growth >20% compared to growth in 2% glucose medium (Table 2). Tolerance to weak acids was good for all strains as at least 80% relative metabolic activity was recorded at the highest tested concentrations for all tested weak acids (Table 2). *T. delbrueckii* 167TD and *P. kudriavzevii* 230PK showed highest tolerance to furfural, with recorded values of about 40% relative metabolic activity at 15 mM furfural (1.44 g l^{-1}), while the other strains showed less than 10% activity. Nevertheless, at 5mM, only *M.pulcherrima* 230MP and *T. delbrueckii* 167TD, showed considerable decrease in activity (30% decrease). Both *S. cerevisiae* strains and *W. anomalus* 061P AG2 showed the best tolerance to vanillin with recorded values of 90-100% activity at 5 mM (0.76 g/l), respectively, while the other strains showed at least 50% reduction (Table 2). No growth was recorded at 10 mM vanillin for any of the strains.

Bioethanol production under high gravity and lignocellulosic hydrolysate stress

Ethanol yield was recorded in 25% glucose fermentation medium. It was observed that *S. cerevisiae* and *W. anomalus* PA G2 yielded the highest ethanol concentrations (g ethanol/g glucose), up to the theoretical maximum of 50% (Table 3) which corresponds to 14% v/v ethanol. The other yeast isolates yielded 26-38% ethanol corresponding to 9-12% v/v. Similar trends were observed for the lignocellulosic hydrolysate fermentations. There was no difference in ethanol yield in the presence and absence of inhibitors for all isolates, except for *M. pulcherrima* 230MP which did not yield any ethanol under inhibitor stress (Table 3). *S. cerevisiae* and *W. anomalus* PA G2 yielded the highest ethanol concentrations up to 61% of the total C6 sugars in the medium. Whereas both *S. cerevisiae* strains reached this value within 22 h (the first sampling point), *W. anomalus* PA G2 needed more time both in the absence (within 42h) and the presence (within 88h) of inhibitors (Table 3 and Fig. 1). At the time point with the maximal ethanol yield, about 10% of the xylose has been consumed by *S. cerevisiae*, probably because it was consumed for growth (Fig. 1, details not shown). *W. anomalus* PA G2 consumed 17% of the xylose in the absence of inhibitors and only 8% in the presence of inhibitors. The greatest decrease of xylose concentration was observed when glucose was still present and before the maximal ethanol yield was achieved (Fig. 1).

Table 3: Maximal ethanol yield (%, g ethanol/g glucose) and the time (h) needed to reach this maximum for six selected yeast isolates in the presence of 25% glucose and the presence and absence of inhibitors[(a)] (with a mixture of 5.8% C6 and 6.7% C5 sugars) related to lignocellulosic hydrolysates.

		High gravity		**Lignocellulosic fermentation**			
		25% glucose		**Without inhibitors**		**With inhibitors**	
Isolate	**Species**	Max. EtOH (%)	Time (h)	Max. EtOH (%)	Time (h)	Max. EtOH (%)	Time (h)
EtOH Red	*Saccharomyces cerevisiae*	49	114	54	19	56	19
SCOak50	*Saccharomyces cerevisiae*	50	52	62	22	61	22
ST1312/239 MP	*Metchnikowia pulcherrima*	38	119	39	42	0	42
ST1312/061 PA G2	*Wickerhamomyces anomalus*	50	127	55	41	59	88
ST1312/167 TD	*Torulaspora delbrueckii*	31	120	42	42	42	42
ST1312/230 PK	*Pichia kudriavzevii*	26	138	43	22	45	22

[(a)] 1.24 g l^{-1} HMF, 0.4 g l^{-1} furfural, 2.3 g l^{-1} acetic acid, 0.44 g l^{-1} formic acid, 0.87 g l^{-1} levulinic acid and 0.04 g l^{-1} vanillin

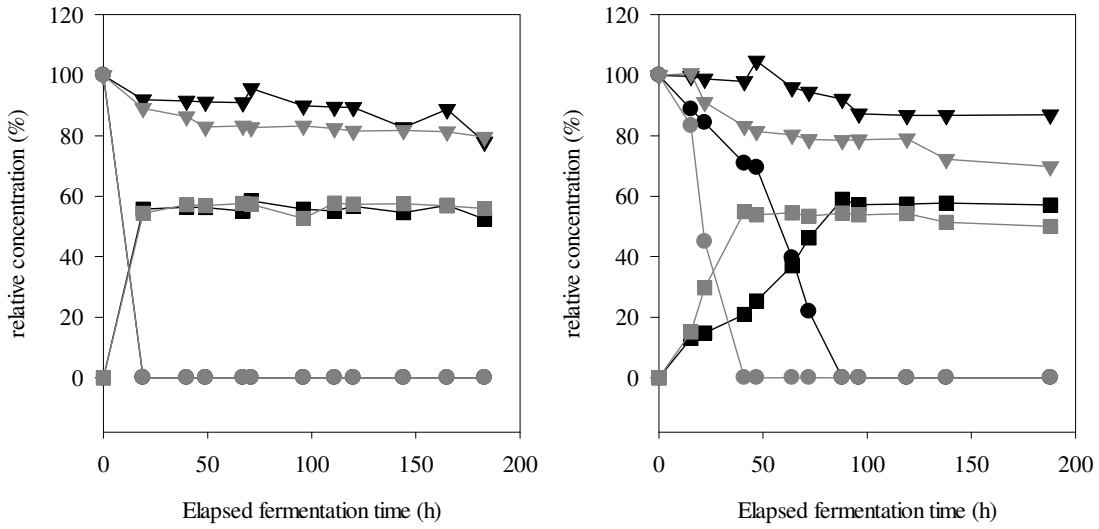

Fig. 1: Consumption of glucose (circles) and xylose (triangles) and production of ethanol (squares) in the presence (black) and absence (grey) of inhibitors for *S. cerevisiae* Ethanol Red (left) and *W. anomalus* PAG2 (right). Glucose and xylose values are relative to their respective values at the start of the fermentation, ethanol values are relative to the total C6 sugar content at the start. In case of Ethanol Red (left) the decrease of glucose is identical in the presence and absence of inhibitors and only the curve in the absence is visualized. Theoretically, 51g ethanol can be obtained from 100g C6 sugars (51%). The inhibitors concentrations are 1.24 g l^{-1} HMF, 0.4 g l^{-1} furfural, 2.3 g l^{-1} acetic acid, 0.44 g l^{-1} formic acid, 0.87 g l^{-1} levulinic acid and 0.04 g l^{-1} vanillin.

Discussion

Currently, *S. cerevisiae* is the organism of choice for fermentation processes producing ethanol [5]. However, bioethanol production from high sugar concentration feedstocks (such as maize, sugar beet) and lignocellulosic hydrolysates confronts the yeast with new challenges which were previously not encountered in any of the known fermentations [29, 38]. These include high ethanol tolerance and osmotolerance in case of high sugar feedstocks and tolerance to osmotic stress and a myriad of inhibitors in case of lignocellulosic hydrolysates. Also, the fermentation of xylose which cannot be performed by non-genetically engineered *S. cerevisiae* is important in the latter case. Exploiting the natural diversity of yeasts might expand the yeast strain repertoire with suitable characteristics for bioethanol fermentation. Some studies previously investigated the potential of non-*S. cerevisiae* yeasts. Huang et al. [16] and Cho et al. [8] showed that *Scheffersomyces stipitis*, a xylose fermenting yeast, had a poor growth rate when inhibitors were present. Blomqvist et al. [6] studied a *Dekkera bruxellensis* strain with better tolerance to inhibitors compared to a *S. cerevisiae* strain, and both strains had a comparable ethanol yield. Delgenes et al. [9] compared the effects of inhibitors on

ethanol production by *S. cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis* and *Candida sheatae*. Zha et al. [36] isolated *Wickerhamomyces anomalus* strains from grass silage that were tolerant to inhibitors. In this study we investigated yeasts isolated from a less commonly studied habitat, i.e. plant nectar and sugar beet thick juice (an intermediate product of sugar production stored in tanks) for their potential to tolerate osmostress, ethanol and HMF as lignocellulosic hydrolysate inhibitor and for their fermentation capacity. Nectar and sugar beet thick juice are known to house several yeast species which are less commonly isolated from typical habitats such as water or soil [2, 26]. In addition, these environments are rather distinct from typical habitats as they contain high concentrations of sugars and/or a myriad of different sugars [12, 26] and are therefore microbially less diverse compared to other environmental habitats.

Our results, as summarized in Table 1, showed that *Metchnikowia reukauffii*, *Hanseniaspora uvarum* and *Starmerella bombicola* isolates, isolated from nectar, showed poor characteristics for bioethanol fermentation, even for low sugar feedstocks, due to a low ethanol tolerance with growth being severely impaired from 5% v/v onwards. Also *Citeromyces matritensis* isolates isolated from sugar beet thick juice showed poor ethanol tolerance. Also, the low HMF tolerance of these four species would impede their use in the fermentation of lignocellulosic hydrolysates. *Candida bombi* isolates from nectar, however, showed a very good osmotolerance (up to 70%) and HMF tolerance (up to 7 g l^{-1}). However, its low ethanol tolerance might impede its use in bioethanol fermentation of first generation and even of lignocellulosic hydrolysates as ethanol concentrations of 5% are readily obtained during fermentation. Similar conclusions can be drawn for three out of four *Torulaspora delbrueckii* isolates, however, they have a lower tolerance to HMF than *C. bombi*. One *T. delbrueckii* showed better ethanol and HMF tolerance and was therefore selected for further experiment. The most promising species is *Wickerhamomyces anomalus*. Although its osmotolerance and HMF tolerance to high concentrations is generally lower than *C. bombi* isolates, it is still good at relevant concentrations for bioethanol production. In addition, ethanol tolerance is much better than *C. bombi* with growth still recorded up to 11% ethanol. *W. anomalus* isolate PA G2 also performed best on xylose (Table 2). This suggests that *W. anomalus* might be interesting for the fermentation of xylose in lignocellulosic hydrolysates. Zha et al. [36] previously identified *W. anomalus* isolates as good candidates for

lignocellulosic hydrolysate fermentation as they showed good resistance to HMF and other inhibitors and growth on xylose as carbon source. In addition, *W. anomalus* might be an interesting species in unsterile fermentations due to its killer activity and biocontrol activity which outcompetes other microorganism during fermentation, such as lactic acid bacteria or other yeasts [30].

Next to tolerance to fermentation inhibitors ethanol yield is also an important parameter. Therefore, we conducted fermentation experiments under controlled conditions using Bioflo bioreactors and measured glucose, xylose and ethanol during the course of the experiment. A high sugar medium containing 25% glucose and an artificial lignocellulosic hydrolysate medium was used to test 4 strains selected based on their tolerance. Two *S. cerevisiae* strains were included as reference, since this species is known for its excellent ethanol yield [33]. One strain is used as industrial bioethanol strain, the other strain is a natural strain isolated from oak and not domesticated for use in fermentation processes. The concentrations of the individual inhibitors in the lignocellulosic hydrolysate medium were similar to the concentrations tested in the liquid assay that not impaired metabolic activity. Our results confirmed the excellent ethanol yield of *S. cerevisiae* compared to other species (Table 3). Both strains yielded an ethanol concentration close to the theoretically maximal yield based on C6 sugars (51%), while the ethanol yield for the selected *M. pulcherrima*, *T. delbrueckii* and *P. kudriavzevii* strains ranged from 26-38%. The selected *W. anomalus* strain also yielded 50% ethanol per g glucose. This is in agreement with the study of Passoth et al. [30] that showed that *W. anomalus* has a good ethanol yield. Both *S. cerevisiae* and *W. anomalus* also showed good ethanol tolerance during these fermentation (up to 14%v/v ethanol), which is essential in high gravity fermentation. Similar trends were observed in the lignocellulosic hydrolysate medium without inhibitors containing about 20% total sugars. Except for *M. pulcherrima* which yielded no ethanol in the presence of inhibitors, no effect of the presence of inhibitors on the ethanol yield was observed for any of the strains. Both *S. cerevisiae* strains performed equally good with the oak derived strain even more tolerant to HMF, showing that good bioethanol production strains for both first and second generation biomass might be omnipresent in nature. In contrast to the 25% glucose fermentation, ethanol yield was above the theoretical value of 51%. Conversion of inhibitors to ethanol is unlikely explaining this, because this is also observed in the absence of inhibitors. Conversion of C5 sugars to ethanol might explain this,

however, this is not expected for *S. cerevisiae* that also showed increased ethanol yield. The ethanol yield of the *W. anomalus* strain was similar to that of both S. *cerevisiae* strains, however, the presence of inhibitors decreased the rate of ethanol production. This shows that *W. anomalus* might be interesting as bioethanol production strain for lignocellulosic hydrolysates if it is able to ferment xylose to ethanol. However, when pregrown on glucose it was shown that xylose was consumed along with glucose consumption, but once glucose was depleted xylose consumption decreased. This interrupted xylose consumption of *W. anomalus* was also observed by Zha et al. [36] and Kurtzman [23] and was contributed to the lack of oxygen present. This might suggest that xylose was not converted to ethanol during the first stage either, but rather used for aerobic growth. Indeed, the ethanol yield based on C6 sugars was similar to both *S. cerevisiae* strains which do not ferment xylose. Pregrowth of *W. anomalus* on 10% xylose did not make any difference in ethanol production and xylose or glucose consumption during fermentation compared to pregrowth on 10% glucose (data not shown). Similar ethanol yields were obtained and only 10% of xylose was consumed.

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Figure caption

Fig. 1 Consumption of glucose (circles) and xylose (triangles) and production of ethanol (squares) in the presence (black) and absence (grey) of inhibitors for *S. cerevisiae* Ethanol Red (left) and *W. anomalus* PAG2 (right). Glucose and xylose values are relative to their respective values at the start of the fermentation, ethanol values are relative to the total C6 sugar content at the start. In case of Ethanol Red (left) the decrease of glucose is identical in the presence and absence of inhibitors and only the curve in the absence is visualized. Theoretically, 51g ethanol can be obtained from 100g C6 sugars (51%). The inhibitors concentrations are 1.24 g l^{-1} HMF, 0.4 g l^{-1} furfural, 2.3 g l^{-1} acetic acid, 0.44 g l^{-1} formic acid, 0.87 g l^{-1} levulinic acid and 0.04 g l^{-1} vanillin.