Electronic Journal of Biotechnology ISSN: 0717-3458
Electron. J. Biotechnol.
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue3-fulltext-8 *RESEARCH ARTICLE*

Inhibitor tolerance and flocculation of a yeast strain suitable for second generation bioethanol production

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Received January 23, 2012 / Accepted April 16, 2012
Published online: May 15, 2012

Abstract

Background: Robust second generation bioethanol processes require microorganisms able to ferment inhibitory lignocellullosic hydrolysates. In this study, the inhibitor tolerance and flocculation characteristics of *Saccharomyces cerevisiae* CCUG53310 were evaluated in comparison with *S. cerevisiae* CBS8066. **Results:** The flocculating strain CCUG53310 could rapidly ferment all hexoses in dilute acid spruce hydrolysate, while CBS8066 was strongly inhibited in this medium. In synthetic inhibitory media, CCUG53310 was more tolerant to carboxylic acids and furan aldehydes, but more sensitive than CBS8066 to phenolic compounds. Despite the higher tolerance, the increase in expression of the *YAP1*, *ATR1* and *FLR1* genes, known to confer resistance to lignocellulose-derived inhibitors, was generally smaller in CCUG53310 than in CBS8066 in inhibitory media. The flocculation of CCUG53310 was linked to the expression of *FLO8*, *FLO10* and one or more of *FLO1*, *FLO5* or *FLO9*. Flocculation depended on cell wall proteins and Ca^{2+} ions, but was almost unaffected by other compounds and pH values typical for lignocellulosic media. **Conclusions:** *S. cerevisiae* CCUG53310 can be characterised as being very robust, with great potential for industrial fermentation of lignocellulosic hydrolysates relatively low in phenolic inhibitors.

Keywords: biofuel, furfural, lignocellulose, phenolic inhibitors, *Saccharomyces cerevisiae*

INTRODUCTION

With depletion of fossil fuels and an increase in environmental awareness, there is a strong drive towards developing bio-based fuels. The most widely used alternative today is bioethanol, most of which is now produced from corn, wheat and sugar cane. However, the use of these starch- and sugar-based resources is controversial, as they are also used as animal feed or staple diet for the growing population of the world (Pimentel et al. 2009). Lignocellulosic materials, that are still low in price and present in abundant amounts, are raw material for second generation bioethanol production that do not affect the food market, and may provide a significantly better degree of sustainability (Solomon et al. 2007).

To achieve an effective production of second generation biofuels, that is, biofuels derived from lignocellulosic crops, it is important to maintain a high fermentation rate. Lignocellulosic materials are recalcitrant, and during pre-treatment and hydrolysis, by-products may be formed that are inhibitory for most microorganisms, such as carboxylic acids, furan aldehydes and phenolic compounds (Klinke et

al. 2004). These may slow down or even stop the fermentation, undermining the feasibility of the process. The inhibitor amounts present in a given hydrolysate depend on both the source of the raw material and the pre-treatment and hydrolysis methods (Klinke et al. 2004).

The preferred and most widely used microorganism for industrial production of bioethanol is *Saccharomyces cerevisiae*. Some *S. cerevisiae* strains are capable of *in situ* detoxification of toxic hydrolysates. However, this requires rather low concentration of the inhibitors, together with a high concentration of biomass (Taherzadeh et al. 1999). The lower inhibitor concentration can be accomplished using continuous or fed batch cultivation, while a higher cell concentration can be achieved by cell immobilisation or cell recycling (Brandberg et al. 2005).

In the search for a reliable and cost effective system for ethanol production from lignocellulosic material, the use of flocculating yeast for fermentation of hydrolysates has been shown to be promising regarding both inhibitor tolerance (Purwadi et al. 2007), resistance against bacterial contamination (Tang et al. 2006) and cell retention in the bioreactor while obtaining a cell-free product (Verstrepen et al. 2003; Brandberg et al. 2005; Zhao and Bai, 2009). Flocculating yeast strains have repeatedly been shown to perform very well in inhibitor tolerance studies (Martín and Jönsson, 2003; Brandberg et al. 2004; Brandberg et al. 2005; Purwadi et al. 2007), and among five recombinant xylose-fermenting yeast strains, a flocculating strain was found to be the best (Matsushika et al. 2009). Flocculation of yeast cells has been shown to be dependent on flocculins, lectin-like proteins in the cell wall of the yeast cells encoded by genes in the *FLO*-gene family, which bind to carbohydrates present in the cell wall of neighboring yeast cells. Dissolved sugars and other compounds may interfere with these interactions and inhibit the flocculation. This is the case for most brewery yeasts, where the flocculation is initiated only upon depletion of the fermentable sugars (Verstrepen et al. 2003).

The flocculating yeast *S. cerevisiae* CCUG53310 has been shown to ferment toxic hydrolysate in both batch and continuous mode (Purwadi et al. 2007; Dehkhoda et al. 2009) and perform better than other investigated strains regarding various environmental stresses (Albers and Larsson, 2009). A better understanding of factors influencing flocculation for this strain will be important for further optimisation of industrial applications using CCUG53310 and flocculating yeasts in general.

In this study, the robustness of the flocculating yeast *S. cerevisiae* CCUG53310 is characterised with respect to its flocculation mechanisms, fermentation performance in lignocellulose hydrolysate, tolerance to different inhibitors, and expression of genes known to confer flocculation ability and resistance to inhibitors. The results are compared with the non-flocculating yeast *S. cerevisiae* CBS8066. Implications regarding tolerance mechanisms and industrial application of *S. cerevisiae* CCUG53310 are discussed.

MATERIALS AND METHODS

Yeast strains and media

The flocculating yeast strain *S. cerevisiae* CCUG53310, registered at the Culture Collection in University of Gothenburg (Sweden), was used in all experiments. The strain originates from an ethanol plant (Domsjö Fabriker AB, Örnsköldsvik, Sweden) (Purwadi et al. 2007). The diploid wild-type strain *S. cerevisiae* CBS8066, obtained from Centraalbureau voor Schimmelcultures (Delft, the Netherlands) was used as a reference strain. Strains were maintained on YEPD agar plates (10 g/l of yeast extract, 20 g/l of soy peptone and 20 g/l of D-glucose as an additional carbon source).

Cultures for flocculation and hydrophobicity tests were grown on YEPD medium containing yeast extract (10 g/l), peptone (20 g/l) and glucose (20 g/l). The growth medium used for batch cultivations was a defined glucose medium used previously for cultivation of the same yeast strains (Purwadi et al. 2007). Inhibitory media for the batch cultivations were chosen to cover the major groups of inhibitors found in lignocellulosic hydrolysates, namely carboxylic acids, furan aldehydes and phenolic compounds. The defined inhibitor media was based on the defined glucose medium, with addition of different inhibitors. All of the media, with the exception of the hydrolysate medium, had a final glucose concentration of ~21-23 g/l. The inhibitor concentrations of the furan aldehydes medium were set to 2.0 g/l 5-hydroxymethylfurfural and 1.5 g/l furfural, the carboxylic acids medium contained 200 mM each of acetic, formic and levulinic acid (12.0, 9.2 and 23.2 g/l respectively), pH adjusted to 5.5 with

concentrated NaOH, and the phenolics medium contained 2 mM vanillin, 1.5 mM guaiacol and 1.5 mM catechol.

The hydrolysate used in this work was produced from SO_2-impregnated spruce chips treated at pH 2.0, 18 bar pressure for 5-7 min. The hydrolysate was stored refrigerated at low pH (~pH 2) until use. Immediately before use, pH was adjusted to 5.5 with concentrated NaOH and the hydrolysate was autoclaved. Salts, trace metals, vitamins and ergosterol were added to the same concentration as in the defined glucose medium. Due to the dilution with the pre-culture, the final concentrations in the hydrolysate medium were approximately 60% of the initial, with glucose contents of 9.2 ± 0.2 g/l, mannose 12.5 ± 0.2 g/l, galactose 2.5 ± 0.0 g/l, xylose 5.2 ± 0.1 g/l, arabinose 1.7 ± 0.0 g/l, acetic acid 2.2 ± 0.1 g/l, furfural 0.19 ± 0.02 g/l, 5-hydroxymethylfurfural 0.79 ± 0.01 g/l, catechol 0.03 ± 0.01 g/l and vanillin 0.08 ± 0.01 g/l.

Flocculation trials

Cells were propagated aerobically on 100 ml YEPD medium in 250 or 300 ml cotton-plugged Erlenmeyer flasks in a shaker bath (125-130 rpm) at 30ºC. Flocculation trials were performed with stationary phase cells. The cells were harvested 48 hrs after inoculation by centrifugation for 5 min, followed by deflocculation in 250 mM EDTA. While suspended in EDTA the cells were heat killed (60ºC, 5 min) to avoid metabolism of the substances tested. This treatment did not affect the flocculation ability. The cells were washed with 30 mM EDTA to ensure complete dispersion, before being washed twice with deionised water. The flocculation test used was a micro-flocculation test similar to the one described by Soares and Mota (1997), with slight modifications. The cell concentration was determined by counting in a Bürker chamber and the suspension was diluted to a concentration of approximately 1 x 10^8 cells/ml in citrate buffer (50 mM, pH 4.5) containing 4 mM $CaCl_2$ and various concentrations of the compounds tested. 2 ml of the suspension were placed in a 12 ml round bottom tube and vortexed vigorously. The tubes were thereafter placed at an angle of approximately 30º on an orbital shaker and agitated at 150 rpm and 25ºC, for 4 hrs to ensure equilibrium. A sample of 200 µl was taken from just below the meniscus after the tubes had been left unagitated in a vertical position for 30 sec. The sample was dispersed in 800 µl of 100 mM EDTA solution and the cell concentration was measured as OD_{600}. The flocculation is presented as the degree of flocculation = (1- free cells/total cells) x 100.

For the test of pH dependence on flocculation, the following buffers were used: Clark and Lubs KCl/HCl (pH 1.0-2.2), citrate (pH 2.0-6.0) and Tris (pH 7.0-9.0). The concentration of all buffers was 50 mM with 4 mM $CaCl_2$. The pH of the buffers was measured prior to cell addition only, it is however known that yeast cells and salt may alter the pH (Stratford, 1989), why the actual pH in the experiments may be slightly changed. The flocculation test was otherwise performed as described above.

The effect of the protease trypsin, 1 mg/ml, was tested using a cell concentration of approximately $1 \cdot 10^9$ cells/ml (determined by counting in a Bürker chamber), in a 0.1 M TRIS-HCl buffer at pH 7.5. The samples were incubated by shaking at 30ºC. Samples were withdrawn at different times, centrifuged and the cells suspended in the flocculation buffer mentioned above, to assay the flocculation.

Hydrophobicity test

The hydrophobicity of cells was tested by the Microbial Adhesion To Hydrocarbons (MATH) assay according to van Mulders et al. (2009) with slight modifications. Cells were propagated aerobically on YEPD medium in 250 or 300 ml cotton-plugged Erlenmeyer flasks in a shaker bath (125-130 rpm) at 30ºC. Stationary phase cells were deflocculated using EDTA and resuspended in 2 ml 0.9% NaCl solution at an OD_{600} of approximately 1. The suspension was overlain with 0.4 ml octane and vortexed at maximum speed for 60 sec. After 10 min a sample of the water phase was taken and OD_{600} was measured. More hydrophobic cells migrate to the octane phase, which lowers the OD_{600} of the water phase. The hydrophobicity is reported as the relative difference between the absorbance before and after vortexing: hydrophobicity = $(1\text{-}OD_{\text{after vortexing}}/OD_{\text{before vortexing}}) \times 100$.

Adhesion test

Adhesive and invasive growth were assayed using a standard plate-washing assay where yeast cells grown overnight in defined glucose medium were streaked on YPD plates and incubated at 30ºC for 4 days. The yeast was thereafter washed off the agar by gentle rinsing followed by rubbing using a gloved finger and the result evaluated by visual inspection.

Quantitative PCR

Yeast cells were grown aerobically in defined glucose medium and cell samples were taken in the exponential phase to study the presence of *FLO* genes, whereas cell samples to study inhibitor tolerance genes were taken after two hours of anaerobic cultivation with inhibitors as described below. 5 ml cell suspension were sprayed on approximately 20 ml ice in 50 ml tubes and centrifuged for 5 min at 4ºC. The pellet was frozen in N_2(l) and stored at -80ºC until RNA extraction. The RNA was extracted with an RNeasy kit (Qiagen) with DNase treatment, according to the manufacturer's protocol. The samples were subjected to reverse transcription and the cDNA was thereafter used for q-PCR. Expression of *TAF10*, *ATR1*, *FLR1*, *YAP1*, *FLO8*, *FLO10* and *FLO11* was quantified using Brilliant® II SYBRGreen QPCR Master Mix, 0.4 µM of forward and reverse primer and 2 µl cDNA. The quantitative PCR (q-PCR) experiments were carried out on a Stratagene Mx3005P instrument. The PCR program started with an initial denaturation for 10 min at 95ºC and amplification using 40 cycles of 30 sec at 95ºC, 1 min at 60ºC followed by 1 min at 72ºC for elongation of the amplicons. A denaturation curve analysis was also included after the last cycle to verify the specificity of the primers. The *TAF10* gene was used as internal reference gene. It was shown to have a stable expression in all samples, since its C_t value did not vary significantly. The primer sequences used in the analysis were designed from the sequences listed in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). The sequences of the oligonucleotide primers were: *ATR1* Forward ATTCTTTGGATGGGGCTCTT; *ATR1* Reverse AGCCCACATTGAATGCTACC; *FLO1* Forward ACTTCTACATCTACTGAAATG; *FLO1* Reverse GTTGGAGTTCTGATGACA; *FLO5* Forward CTAGTGAGGGTTTGATTAC; *FLO5* Reverse AATAACAGTTTCGTCAGTT; *FLO8* Forward AACAAGTGAACCCGCTATGG; *FLO8* Reverse ACTGGAAAAGCTGAGGTGGA; *FLO9* Forward CGGGTTCTTACACATTCA; *FLO9* Reverse GATGGGAGGTTGTTCTTG; *FLO10* Forward CTACACAACACCCACCAACG; *FLO10* Reverse ACGTTGACCCCTTTATGTCG; *FLO11* Forward CGGCTATTCCAACCACAGTT; *FLO11* Reverse AGCCACGCTAGAAGCAGAAG; *FLR1* Forward GCCTGCCTCTGTCTTTGTTC; *FLR1* Reverse ACCAAACAACGGAAAAGCAC; *TAF10* Forward TACCCGAATTTACAAGAAAAGATAAGA; *TAF10* Reverse ATTTCTGAGTAGCAAGTGCTAAAAGTC; *YAP1* Forward TACACGTGATGGCGAGGATA; *YAP1* Reverse CCACTTCATTTTGCTGCTGA. The *FLO1, FLO5* and *FLO9* genes are known to be highly homologous and have previously been reported to be impossible to separate using SYBR green chemistry alone. Therefore, specific hydrolysis probes were designed for these genes based on the *S. cerevisiae* S288c genome sequence. The sequences of the hydrolysis probes were: *FLO1* TTACTGGAACCAACGGCGT; *FLO5* CGGTCACAGTAGTCATCTCAGT; *FLO9* CAATGCTACCACCGACTGATAGAAT.

Data was evaluated using the comparative C_t method: relative gene expression = $(1 + E_{ref})^{Ct_{ref}} / (1 + E_{target})^{Ct_{target}}$, where the efficiencies, E, were calculated from a dilution series of the cDNA template.

Batch cultivation procedures

The batch cultivations were carried out in 250 ml conical flasks, cotton plugged for aerobic cultivation and equipped with rubber stoppers fitted with two stainless steel capillaries and a glass loop trap for anaerobic cultivations. Sterile water was used in the loop traps to permit produced CO_2 to leave the flasks. Cultivations were started with 36 hrs aerobic cultivation of 40 ml defined glucose medium in a shaker bath (125 rpm) at 30ºC. To make reproducible inoculations of the anaerobic cultivations also when using flocculating cells, 80 ml fresh medium of different compositions was added directly to each preculture, giving a total volume of 120 ml with the desired initial concentrations.

Samples for HPLC analysis were taken through one of the steel capillaries. The samples were centrifuged to remove cells and stored at -20ºC until analysis. The specific growth rate was estimated from the glucose consumption, since sedimentation of cells makes sampling of the cell mass very difficult. Yields were calculated at the end of the cultivations, where all biomass could be subjected to dry weight determination.

Analytical methods

Metabolites and inhibitors were quantified by HPLC with an Aminex HPX-87H column (Bio-Rad) at 60ºC eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. A refractive index detector was used for glucose, formic acid, acetic acid, levulinic acid, glycerol, ethanol, furfural and 5-hydroxymethylfurfural, and a UV detector was used for guaiacol, catechol and vanillin. For the hydrolysate samples, an additional Aminex HPX-87P (Bio-Rad) column at 85ºC eluted with ultrapure water at a flow rate of 0.6 ml/min was also used to analyse the glucose, xylose, galactose and mannose concentrations. These compounds were detected using a refractive index detector. The cell dry weight was measured in predried and preweighed Eppendorf-tubes, glass tubes or watch glasses. The cells were washed once with distilled water before drying for approximately 24 hrs at 105ºC. The total carbohydrate content was determined by the colorimetric phenol-sulphuric acid method with a glucose standard. Proteins were extracted by boiling for 10 min in 1 M KOH. The protein content was determined by a modified Biuret method (Verduyn et al. 1990) after cooling on ice. $CuSO_4$ was added to the extracts at a final concentration of 25 mM and after 5 min centrifuged to remove the precipitate of $Cu(OH)_2$. Bovine serum albumin was used as standard and the absorbance of the samples was measured at 550 nm. The RNA content of the cells was determined by degradation in KOH and extraction in perchloric acid. The RNA concentration was subsequently determined spectrophotometrically at 260 nm using average nucleotide data: Mw = 240 g/mol, ε = 10800 $M^{-1}cm^{-1}$.

Statistics, yields and elemental-balance calculations

The biomass and metabolite yields were calculated from the determined concentrations at the end of the fermentations. Produced carbon dioxide was considered to be at the same molar ratio as ethanol and acetate. The biomass composition $CH_{1.76}O_{0.56}N_{0.17}$ (Verduyn et al. 1990) was used in the carbon and redox balance calculations. Ethanol yields corrected for evaporation were calculated by adding the carbon missing from the carbon balance calculations as ethanol and carbon dioxide, and subsequently recalculating the yields. Error intervals shown are ± standard deviation unless otherwise mentioned.

RESULTS AND DISCUSSION

In a previous study, it was concluded that the flocculating yeast *S. cerevisiae* CCUG53310 was more stress tolerant than other, non-flocculating, laboratory strains due to its industrial background (Albers and Larsson, 2009). Its use in industrial circumstances has also been proposed (Purwadi et al. 2007). However, for successful application and optimisation of CCUG53310 in industrial bioethanol production, a deeper understanding of factors influencing both the flocculation and the inhibitor tolerance of the yeast is required.

In this work the strain CCUG53310 has been characterised in comparison with the non-flocculating strain CBS8066, to a higher level of detail. The physiology of CBS8066 has been studied extensively (see *e.g.* Verduyn et al. 1990; Sárvári Horváth et al. 2003; Talebnia and Taherzadeh, 2007) making it a good reference strain.

Fermentation performance in inhibitory media

The ability to ferment toxic hydrolysates is crucial for successful second generation bioethanol production. The composition of sugars and inhibitors in lignocellulosic media vary both with the raw material and the chosen pre-treatment and hydrolysis methods. Therefore it is of great interest to elucidate the reasons for the inhibitor tolerance observed in the flocculating yeast strain CCUG53310 (Purwadi et al. 2007) and to identify potential differences in tolerance against various inhibitors. To investigate this, the flocculating and the non-flocculating strains were cultivated in complete hydrolysate as well as in defined glucose medium with and without selected inhibitors.

When cultivated in defined glucose medium without inhibitors, CCUG53310 and CBS8066 showed similar growth characteristics with regard to glucose consumption and ethanol production (Figure 1a and Table 1). However, the flocculating strain grew at a significantly lower specific growth rate than the non-flocculating strain in the defined glucose medium (Table 1), likely because of mass transfer limitations into the flocs.

The flocculating yeast rapidly consumed the fermentable sugars and produced ethanol at a high yield also in dilute acid spruce hydrolysate medium (Figure 1b and Table 1). Despite the relatively low concentrations of the individual inhibitors, the non-flocculating strain only consumed very little of the fermentable sugars in this medium. This supports previously published data (Purwadi et al. 2007).

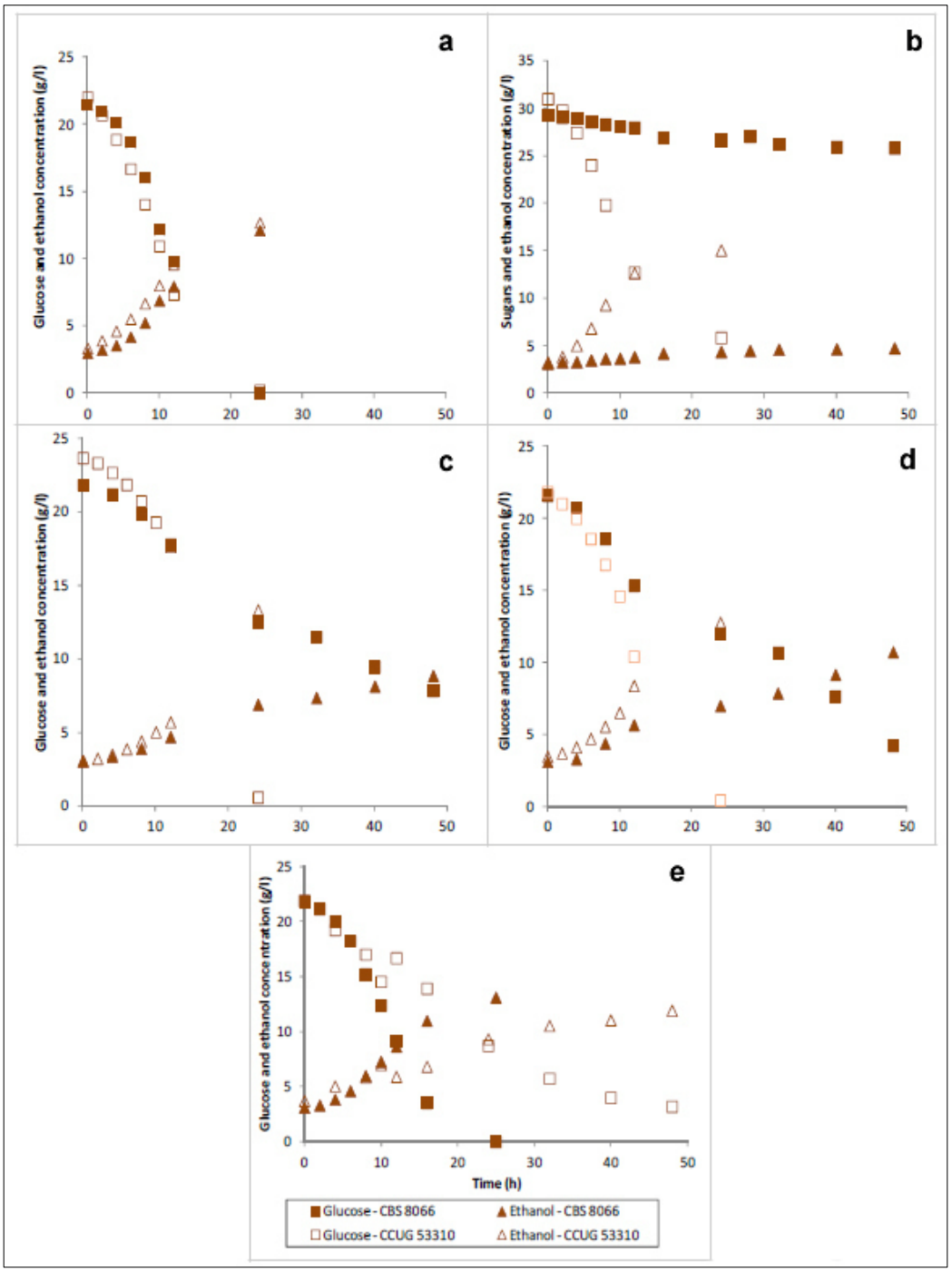

Fig. 1 Sugar consumption and ethanol production in inhibitory media. Cells were cultivated in (a) defined glucose medium, (b) spruce hydrolysate, and defined media containing (c) carboxylic acids, (d) furan aldehydes and (e) phenolic compounds as described in Materials and Methods. Sugar and ethanol concentrations were monitored by HPLC measurements during the fermentation by *S. cerevisiae* CCUG53310 and CBS8066. Presented data are mean values of at least two cultivations per data point.

Table 1. Calculated yields, specific growth rate and macromolecular composition of the biomass in anaerobic batch cultivations.

Medium	Strain	Y_{SE}	Y_{SAce}	Y_{SGly}	$Y_{SBiomass}$	$Y_{SE, corr}$	μ (h^{-1})	Carb (%)	Prot (%)	RNA (%)	R/P (%)
Defined glucose medium	8066	435 ± 10	11 ± 3	44± 2	64 ± 4	441 ± 1	0.35 ± 0.02	37 ± 5	40 ± 4	9 ± 3	22 ± 4
	53310	430 ± 5	0 ± 1	54± 5	62 ± 4	446 ± 2	0.24 ± 0.02	43 ± 2	43 ± 2	9 ± 1	20 ± 2
Hydrolysate	8066	411 ± 5	70 ± 26	24 ± 10	76 ± 37	n/a	n/a	n/a	n/a	n/a	n/a
	53310	466 ± 15	4 ± 5	52 ± 10	72 ± 3	n/a	n/a	40 ± 4	n/a	10 ± 1	n/a
Carboxylic acids	8066	416 ± 48	32 ± 10	73 ± 8	13 ± 7	443 ± 10	0.04 ± 0.01	38 ± 4	45 ± 4	8 ± 1	18 ± 2
	53310	436 ± 29	25 ± 23	64 ± 13	27 ± 16	459 ± 31	0.18 ± 0.03	39 ± 3	46 ± 2	9 ± 1	20 ± 0
Furan aldehydes	8066	432 ± 12	8 ± 2	33 ± 2	30 ± 3	469 ± 2	0.06 ± 0.02	40 ± 2	43 ± 3	6 ± 1	15 ± 1
	53310	435 ± 21	-2 ± 2	33 ± 2	50 ± 2	465 ± 3	0.20 ± 0.01	39 ± 3	40 ± 1	7 ± 0	18 ± 1
Phenolics	8066	459 ± 18	6 ± 4	35 ± 3	63 ± 11	n/a	0.30 ± 0.02	34 ± 2	37 ± 1	9 ± 0	26 ± 1
	53310	429 ± 17	1 ± 3	63 ± 6	35 ± 7	468 ± 22	0.11 ± 0.02	35 ± 2	41 ± 2	8 ± 1	18 ± 1

Values are 95% confidence intervals of the mean (n ≥ 2). Yields are shown in mg product per g consumed hexose, macromolecular composition in % of dry, ash free weight. 8066: Non-flocculating strain CBS8066, 53310: Flocculating strain CCUG53310, Y_{SE}: Ethanol yield on substrate, Y_{SAce}: Acetate yield on substrate, Y_{SGly}: Glycerol yield on substrate, $Y_{SBiomass}$: Biomass yield on substrate, $Y_{SE, corr}$: Ethanol yield corrected for evaporated ethanol, μ: specific growth rate at time of sampling, calculated from consumed glucose, Carb: Cellular carbohydrate content, Prot: Cellular protein content, RNA: Cellular RNA content, R/P: Cellular RNA: protein ratio, n/a: Not applicable/available.

DOI: 10.2225/vol15-issue3-fulltext-8

The consumption of glucose by CCUG53310 was slower in defined media containing carboxylic acids and furan aldehydes than in the absence of these inhibitors, indicating an inhibition effect (Figure 1c-1d). Nevertheless, the flocculating strain was able to consume all the glucose. The non-flocculating strain CBS8066 was significantly slower at consuming the glucose, and in the carboxylic acids medium it could not consume all the sugar. The two strains had similar initial glucose consumption rates, but the glucose uptake rate of CBS8066 decreased throughout the cultivation (Figure 1c-d). Thus, the non-flocculating strain was clearly more affected by the acids and furan aldehydes. The decrease in furfural and 5-hydroxymethylfurfural concentration was also lower for the non-flocculating strain than for the flocculating strain (Table 2).

Table 2. Inhibitor decrease in anaerobic batch cultivations.

Medium	Strain	HMF (%)	Furfural (%)	Vanillin (%)
Furan aldehydes	CBS8066	73 ± 2	100 ± 0	n/a
	CCUG53310	89 ± 10	100 ± 0	n/a
Phenolics	CBS8066	n/a	n/a	89 ± 5
	CCUG53310	n/a	n/a	100 ± 0
Hydrolysate	CBS8066	10 ± 2	67 ± 11	n/a
	CCUG53310	99 ± 2	93 ± 15	n/a

The decrease is shown as the percentage removed from the initial concentration of the inhibitory compound, with 95% confidence intervals (n ≥ 2) of *S. cerevisiae* strains grown in the indicated media. HMF: 5-hydroxymethyl furfural; n/a-not applicable/available.

Interestingly, the rate of glucose consumption of the flocculating yeast strain was lower than that of CBS8066 in the medium containing phenolic inhibitors (Figure 1e). Nevertheless, the removal of vanillin was faster in the flocculating yeast, and also a small decrease in the guaiacol concentration could be observed that was not detected for the non-flocculating strain (data not shown). No decrease in the concentration of catechol was detected in any of the strains (data not shown).

The small effect of most inhibitors on the fermentative capacity of the flocculating strain CCUG53310 shows a general robustness of the strain. In comparison with the non-flocculating strain CBS8066, it was only marginally affected by the presence of furan aldehydes or carboxylic acids in the growth medium. The increased sensitivity of the flocculating strain to the chosen phenolic compounds shows that the inhibitor tolerance is however not universal to all inhibitors.

Expression of genes of importance for inhibitor tolerance

Due to the observed differences in fermentation abilities between the flocculating and non-flocculating strains in the inhibitory media tested, a q-PCR study of known tolerance-linked genes was performed. The expression of the genes *YAP1*, *ATR1* and *FLR1* was determined in the two strains cultivated in the inhibitory media.

These genes were chosen for expression analysis since they are involved in the resistance to phenolic fermentation inhibitors (Sundström et al. 2009). When over expressed, these genes confer resistance to lignocellulose-derived inhibitors (Alriksson et al. 2010). *YAP1* encodes a transcription factor and responds to various different stress conditions. It activates pleiotropic drug resistance (Sundström et al. 2009), is important in the oxidative stress response (Herrero et al. 2008) and has been shown to confer resistance to coniferyl aldehyde, 5-hydroxymethylfurfural and spruce hydrolysate (Alriksson et al. 2010). *YAP1* is also involved in the control of *ATR1* and *FLR1* expression. *ATR1* encodes a membrane transporter protein required for aminotriazole resistance and has been shown to confer resistance to coniferyl aldehyde when over expressed (Alriksson et al. 2010). *FLR1* encodes a plasma membrane transporter protein and has been shown to confer resistance to coniferyl aldehyde and HMF (Alriksson et al. 2010). Thus, differences in inhibitor tolerance could potentially be linked to differences in the expression of these genes between CCUG53310 and CBS8066.

The expression of *YAP1* was increased in both strains when furan aldehydes were present in the medium (Figure 2a-b). The largest increase could be seen in hydrolysate medium for the non-

flocculating strain. For the flocculating strain an increase in *YAP1* expression could be seen in carboxylic acids medium, which was not observed in the non-flocculating strain. This could possibly lead to transcription of other genes important for resistance against carboxylic acids that are not expressed in the non-flocculating strain. Interestingly, the *YAP1* expression was not increased in hydrolysate or phenolics media in the flocculating strain (Figure 2b). In the defined glucose medium, the expression level of *YAP1* in the flocculating strain was approximately 50% higher (relative to *TAF10*) than in the non-flocculating strain. This higher initial level of the gene may be one reason for the better performance of the flocculating strain under the inhibitor stress.

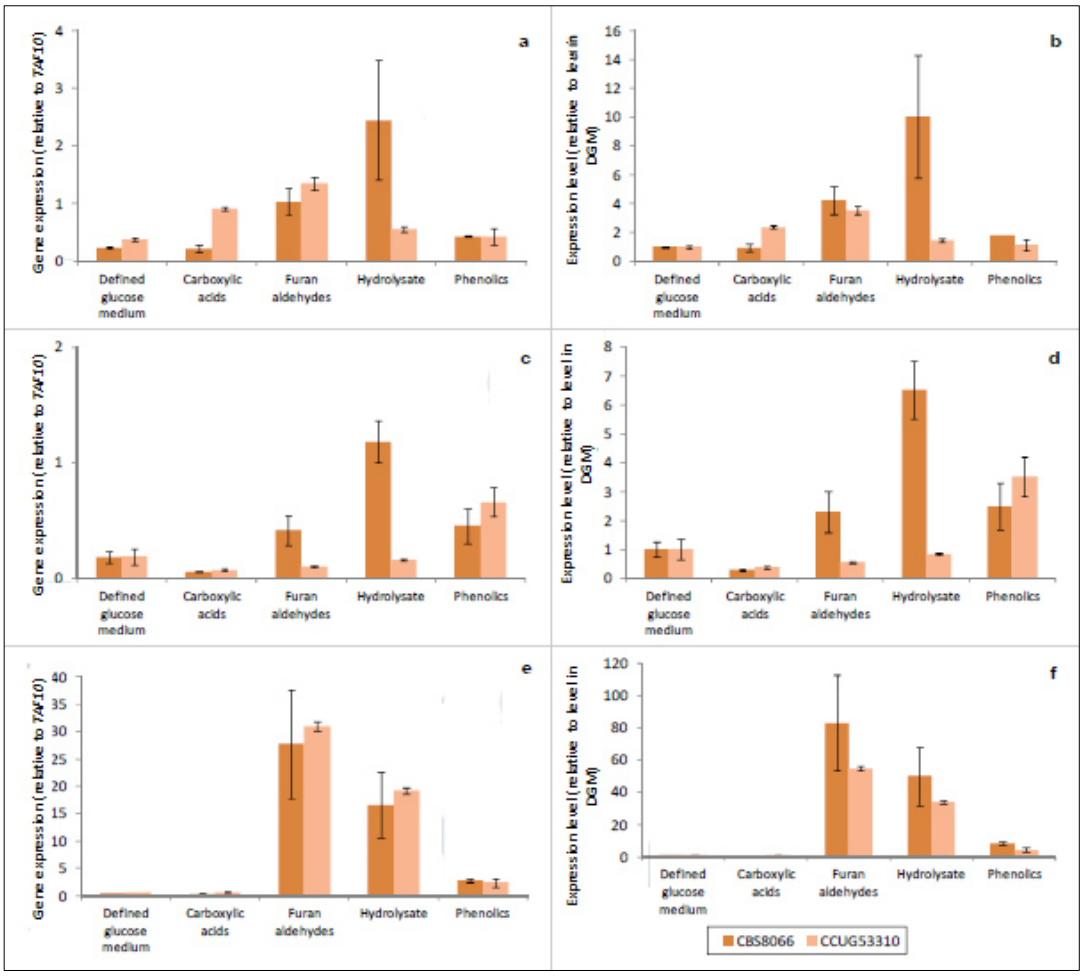

Fig. 2 Expression of *YAP1*, *ATR1* and *FLR1* in defined glucose medium and inhibitory media. The mean expression levels of (a, b) *YAP1*, (c, d) *ATR1* and (e, f) *FLR1*, genes known to confer resistance to lignocellulose-derived inhibitors, were determined by q-PCR in *S. cerevisiae* CBS8066 and CCUG53310 grown in the indicated inhibitory media. The mean expression levels are shown relative to *TAF10* (a, c, e) and after normalisation to the relative expression level in defined glucose medium (b, d, f). Error bars depict two biological replicates.

The expression of *ATR1* correlated with the fermentation performance in the various media. In the non-flocculating strain the expression of *ATR1* was up-regulated in all inhibitory media except carboxylic acids, and especially in the hydrolysate medium (Figure 2c-d). The flocculating yeast strain on the other hand, showed an increased expression of *ATR1* only in the case of phenolic inhibitors. In both strains however, a down regulation of *ATR1* could be seen when acids were present in the medium. The levels of *ATR1* in defined glucose medium were the same in the two strains (Figure 2c), why differences in the *ATR1* expression are likely to arise from how well they can cope with the stress from the inhibitors.

FLR1 was strongly induced by the presence of furan aldehydes in both strains, showing the importance of this gene in the response to the presence of furan aldehydes (Figure 2e-f). An increased expression of the gene was also seen in the presence of phenolic compounds. These results indicate that both furan aldehydes and phenolic compounds can be exported by the Flr1p transporter. The expression level of *FLR1* was unaffected in both strains in medium with carboxylic acids. The level of *FLR1* relative to *TAF10* in defined glucose medium was approximately 60% higher in the flocculating strain. This can, for similar reasons as for *YAP1*, result in a better handling of the stress created from furan aldehydes despite a lower induction of this gene in CCUG53310.

Despite better fermentation performance both *YAP1* and *ATR1* were expressed at lower levels in CCUG53310 than in CBS8066 in the hydrolysate. This may indicate that flocculation indeed leads to a lower stress level. Since *YAP1* is important in the oxidative stress response, this could be interpreted as the flocculating cells containing lower levels of oxidants. However, this remains to be validated.

The unchanged expression or down regulation of both *ATR1* and *FLR1* in the carboxylic acids medium likely owes to the fact that other transporters are important for weak acid transport, such as Fps1p, Pdr12p and Ady2p (Casal et al. 2008).

Macromolecular composition of cells

Changes in the macromolecular composition of the cells give an insight into the physiological state of the cells. It is known that the RNA:protein ratio changes in different growth conditions, and can give an estimate of the growth rate of the cells (Karpinets et al. 2006). In order to investigate the effect of different inhibitor classes on the overall physiology of the flocculating yeast strain, the cellular contents of carbohydrates, proteins and RNA were analysed in cell samples taken in the estimated exponential phase when approximately ¼ to ½ of the sugar was consumed (Table 1). No significant differences could be seen in the RNA:protein ratio when comparing the flocculating yeast strain grown in different media, although the trends showed a lower ratio in inhibitory media. However, although no statistically significant difference could be seen between the two strains when grown in defined glucose medium, the RNA:protein ratio was always lower in the yeast strain that was more inhibited by the respective media (Table 1).

It has been suggested that in an inhibiting growth medium, the protein synthesis becomes more efficient due to increased transcription of cellular maintenance genes and activation of the general stress response (Karpinets et al. 2006). These responses would happen at the expense of growth related genes, leading to a lower specific growth rate. A more efficient protein synthesis would require fewer ribosomes per produced protein. Thus a more inhibiting media would lead to a lowering of the RNA:protein ratio in the cells, which is consistent with our results (Table 1).

For the non-flocculating strain, the RNA:protein ratio was found to have a linear relationship with the growth rate, $R_{RNA:P} = 0.26\ \mu + 0.15$ ($R^2 = 0.75$). A similar correlation has earlier been found in another strain of *S. cerevisiae*, $R_{RNA:P} = 0.26\ \mu + 0.12$ (Karpinets et al. 2006). Although the relationship is known to differ significantly between different strains, this to some extent justifies our estimation of the growth rates. No clear correlation could be identified for the flocculating strain, which can be explained by the more robust nature of the strain leading to smaller differences in both the growth rate and the RNA:protein ratio.

Mechanism and robustness of flocculation

It is important that flocculation is not inhibited by low pH, sugars, acetate, ethanol and other compounds present in lignocellulose hydrolysates, since this would compromise the benefit of flocculation at high gravity fermentation. The dependence of the flocculation on Ca^{2+} and cell wall proteins was shown by treatment of the CCUG53310 cells with the chelating agent EDTA and the protease trypsin, similar to what has been shown for other strains (Ma et al. 2009). Treating flocculating cells with EDTA effectively deflocculated the cells. EDTA acts as a chelating agent, removing Ca^{2+} from the flocs (Stratford, 1989). When Ca^{2+} was removed the cells were unable to bind to each other. Flocs were rapidly reformed after removal of the EDTA and addition of $CaCl_2$ to the cells. CCUG53310 also lost its ability to flocculate after approximately two hours of treatment with trypsin. The results are in agreement with the theory of lectin-like cell wall proteins, flocculins or adhesins, which specifically

bind to carbohydrates in the cell wall of adjacent yeast cells (Touhami et al. 2003; Verstrepen et al. 2003).

Flocculation of *S. cerevisiae* CCUG53310 was inhibited by 1 M mannose (Figure 3), although very weakly compared to what has been reported for other strains (Sieiro et al. 1995). No effect on the flocculation could be observed from glucose, fructose, maltose, sucrose and xylose. Other tested compounds, *i.e*. acetate (0-0.2 M), ethanol (0-30%), furfural (0-0.5 M) did not affect the flocculation. Moreover, the yeast strain showed a very pH tolerant flocculation, with only a minor loss of flocculation at pH below 2.0, likely due to loss of native structure of the flocculins at this pH.

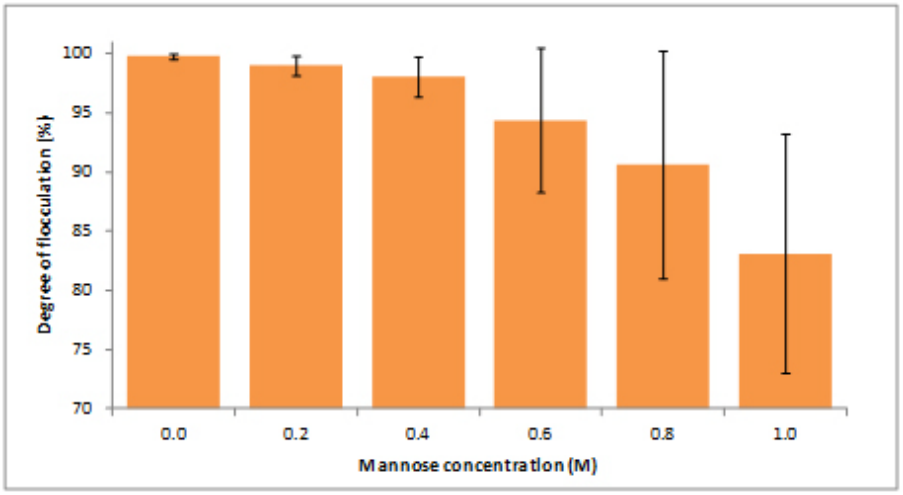

Fig. 3 The effect of mannose on flocculation of *S. cerevisiae* CCUG53310. Error bars show the 95% confidence interval for the mean (n=8).

Interaction between ligands and receptors are often facilitated partly by hydrophobic interactions (van Mulders et al. 2009). The flocculating strain indeed showed significantly higher cell wall hydrophobicity (52.2 ± 3.4%, 95% confidence interval, n=3) compared to the non-flocculating strain (33.3 ± 4.6%). The higher hydrophobicity is likely caused by the flocculins present in the cell wall. In this way these proteins would act in two ways to promote flocculation; by direct binding to carbohydrates and by hydrophobic interactions (van Mulders et al. 2009). The significantly higher hydrophobicity may explain the increased sensitivity to phenolics of the flocculating strain. The hydrophobic cell wall may attract phenolic compounds, thereby creating a significantly higher local concentration around the cells and hence a stronger inhibition. This would also explain the faster removal of vanillin and the decreased concentration of guaiacol in the growth medium observed for the flocculating yeast strain. The increased sensitivity against phenolic compounds is an important factor to consider for fermentation of hydrolysates in industrial settings, since unconverted inhibitors can be accumulated if the medium is recycled.

To clarify the genetic basis of flocculation, the expression of the *FLO* gene family was determined in the two strains using q-PCR. *FLO8* encodes a transcription factor that controls the expression of flocculin genes (van Mulders et al. 2009). In most laboratory strains it contains a mutation which introduces a stop codon and hence a truncated version of the transcription factor, leading to abolished transcription of the other *FLO* genes (Liu et al. 1996). The expression of *FLO8*, relative to *TAF10*, was at the same level in the two tested strains (Figure 4). The expression level of *FLO10*, which has been shown to confer weak flocculation when over expressed (van Mulders et al. 2009), was approximately twice as high in CCUG53310 than in the non-flocculating strain (Figure 4).

We were unfortunately unable to determine the individual expression of the major flocculation-conferring genes *FLO1*, *FLO5* and *FLO9* using SYBR Green chemistry with the primers used, nor with the specific hydrolysis probes designed using the *S. cerevisiae* S288C genome sequence. These

genes are known to be very homologous, display considerable sequence variability in different strains and evolve rapidly (Smukalla et al. 2008). Q-PCR analysis using the *FLO5* primers and SYBR Green indeed gave significantly higher fluorescence response in the flocculating yeast strain. These results are indicative of a significantly higher expression of one or more of *FLO1*, *FLO5* and *FLO9*, since all these include several possible targets for the *FLO5* primers.

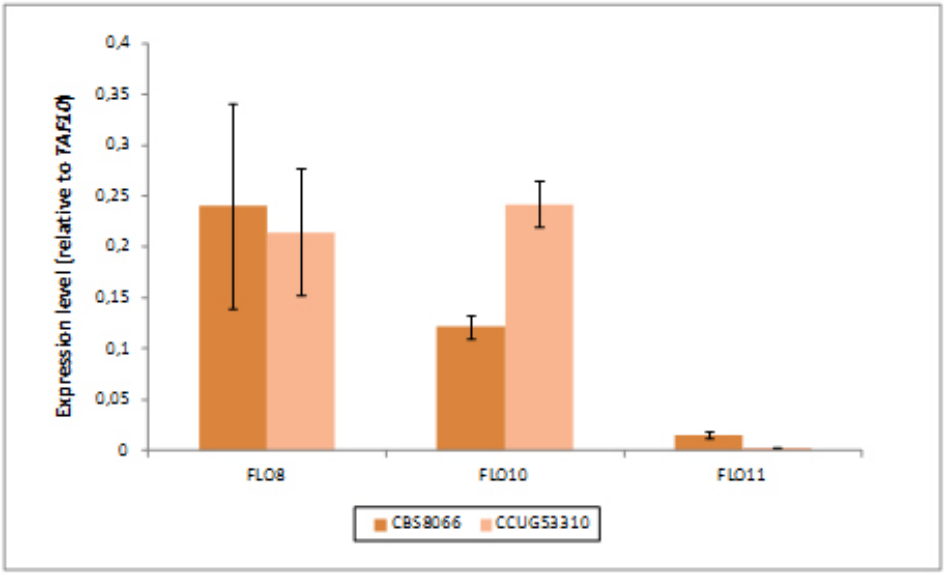

Fig. 4 Expression of *FLO* genes. The mean expression levels of *FLO8*, *FLO10* and *FLO11* were determined in exponential phase cells of *S. cerevisiae* CBS8066 and CCUG53310 by q-PCR as described in Materials and Methods, and are shown relative to the reference gene *TAF10*. Error bars depict two biological replicates.

Since the same gene family that is responsible for flocculation is also involved in adhesive and invasive growth (van Mulders et al. 2009), these phenotypic traits were investigated for the two yeast strains. The flocculating strain adhered somewhat more strongly to the agar surface in the plate washing assay, requiring more rinsing to remove the superficial cells. No invasive growth could be observed for the flocculating strain. On the other hand, the non-flocculating strain showed less adhesive growth but significant invasive growth into the agar. The expression of *FLO11* was approximately five times higher in CBS8066 than in CCUG53310 (Figure 4). *FLO11* (also known as *MUC1*) is known to confer invasive growth (Lambrechts et al. 1996). The higher expression of *FLO11* in the non-flocculating strain provides an explanation for the observed invasive growth and is similar to what is seen in the strain Σ1278b, where the only flocculin expressed is Flo11p (Guo et al. 2000).

The higher expression level of *FLO10* may partially explain the flocculation of CCUG53310. However, *FLO8* expression in this strain most likely also leads to the expression of one or more of *FLO1*, *FLO5* or *FLO9*, which together with hydrophobic interactions, gives rise to the flocculating phenotype. On the other hand, similar *FLO8* expression in CBS8066 did not lead to flocculation. This is also the case for *S. cerevisiae* Σ1278b, which has been shown to lack Flo8p binding sites in the promoter regions of *FLO1* (Fichtner et al. 2007). From the phenotypic characterization it is clear that Ca^{2+} ions are required which may be a problem in industrial conditions. Considering the high amounts of mannose needed to deflocculate the cells of this strain and the insensitivity to other compounds, the flocculation should still be very robust at concentrations that can be expected even in very high gravity spruce hydrolysates.

These new insights into the requirements for flocculation and resistance to inhibitors of CCUG53310 are beneficial to 2nd generation bioethanol production, since one of the major problems to overcome has been the toxicity of lignocellulosic hydrolysates. Using this flocculating strain not only provides the necessary inhibitor resistance but also enables higher cell loadings and easier separation of the product.

CONCLUDING REMARKS

S. cerevisiae CCUG53310 proved to be very robust and tolerant against most inhibitors present in spruce hydrolysate with the exception of phenolic inhibitors. Flocculation was dependent on Ca^{2+} and cell wall proteins but was only weakly inhibited by the presence of mannose. Collectively, our results showed that CCUG53310 is a suitable strain for fermentation of mannose-rich spruce hydrolysates with moderate levels of phenolic compounds.

ACKNOWLEDGMENTS

The authors thank Dr. Tomas Brandberg (SEKAB AB, Sweden) for providing the spruce hydrolysate. We are grateful to our colleagues in the Industrial Biotechnology group, Chalmers, for critical reading and commenting of the manuscript.

Financial support: This project was financed by the Swedish Energy Agency, project no. P13872-2.

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How to reference this article:

WESTMAN, J.O.; TAHERZADEH, M.J. and FRANZÉN, C.J. (2012). Inhibitor tolerance and flocculation of a yeast strain suitable for second generation bioethanol production. *Electronic Journal of Biotechnology*, vol. 15, no. 3. http://dx.doi.org/10.2225/vol15-issue3-fulltext-8