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Ethanol production from selected lignocellulosic hydrolysates by genome shuffled strains of *Scheffersomyces stipitis*

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ABSTRACT

Two genome-shuffled *Scheffersomyces stipitis* strains, GS301 and GS302, exhibiting improved tolerance to hardwood spent sulphite liquor, were tested for growth and fermentation performance on three wood hydrolysates: (a) steam-pretreated enzymatically hydrolyzed poplar hydrolysate from Mascoma Canada, (b) steam pretreated poplar hydrolysate from University of British Columbia Forest Products Biotechnology Laboratory, and (c) mixed hardwoods pre-hydrolysate from FPInnovations (FPI). In the FPI hydrolysate, the wild type (WT) died off within 25 h, while GS301 and GS302 survived beyond 100 h. In fermentation tests, GS301 and GS302 completely utilized glucose and xylose in each hydrolysate and produced 0.39–1.4% (w/v) ethanol. In contrast, the WT did not utilize or poorly utilized glucose and xylose and produced non-detectable to trace amounts of ethanol. The results demonstrated cross tolerance of the mutants to inhibitors in three different wood hydrolysates and reinforced the utility of mating-based genome shuffling approach in industrial yeast strain improvement.

1. Introduction

During the pretreatment of lignocellulosic biomass, a variety of inhibitory compounds are formed. The types and concentrations of the inhibitors may vary depending on the substrate and pre-treatment process applied (Almeida et al., 2007; Lohmeier-Vogel et al., 1998).

The pentose-fermenting yeasts *Scheffersomyces stipitis*, *Pachysolen tannophilus* and *Candida shehatae* ferment xylose but are susceptible to the inhibitors generated during the pretreatment of plant biomass (Bajwa et al., 2009, 2010; Lohmeier-Vogel et al., 1998). Among the inhibitors, acetic acid, furfural, hydroxymethylfurfural (HMF) and some phenolic compounds are considered to be the most toxic (Klinke et al., 2004; Richardson et al., 2011). These inhibitors may act synergistically to greatly reduce yeast growth, viability, and fermentation (Bajwa et al., 2009; Van Zyl et al., 1988; Lohmeier-Vogel et al., 1998).

Various approaches have been investigated to address the adverse effect of inhibitors in lignocellulosic hydrolysates, including altering the pretreatment conditions, detoxifying the hydrolysate or developing yeast strains with improved inhibitor tolerance with the goal of improving ethanol production from lignocellulosic hydrolysates (Parawira and Tekere, 2011; Richardson et al., 2011; Zaldivar et al., 2001). The first two approaches add to the processing cost. The most advantageous and cost-effective strategy is to develop yeast strains with improved inhibitor tolerance. Some yeast strains have been designed, adapted, or mutated to tolerate pretreatment-derived inhibitory compounds (Bajwa et al., 2009, 2010; Ho et al., 1998; Jonsson et al., 1998; Parekh et al., 1987). However, the yeast strains obtained thus far are not robust enough for efficient ethanol production from lignocellulosic hydrolysates.

Recently, random mutagenesis followed by cross mating based genome shuffling was used to improve the tolerance of *S. stipitis* towards hardwood spent sulphite liquor (HW SSL). This approach led to considerable improvement in tolerance to HW SSL in the selected mutant strains which retained their growth and fermenting ability (Bajwa et al., 2010). Lignocellulosic substrates other than HW SSL also contain many of the same inhibitors, albeit in different concentrations. Thus, it is hypothesized that these HW SSL tolerant *S. stipitis* strains should also be tolerant to the inhibitors in other lignocellulosic hydrolysates. In the present study, the growth and fermentation performance of two genome shuffled *S. stipitis* strains (GS301 and GS302) were tested in three other xylose-rich

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wood hydrolysates with different sugar and inhibitor concentrations and compared with that of the *S. stipitis* wild type (WT). The results showed that the genome shuffled strains also exhibited improved tolerance to inhibitors and fermented the major sugars in other lignocellulosic hydrolysates. This reinforces the utility of the genome shuffling approach in the genetic improvement of multi-genic characters in *S. stipitis*.

2. Methods

2.1. Yeast strains, culture maintenance and inoculum preparation

Scheffersomyces (*Pichia*) *stipitis* NRRL Y-7124 (NRC 2548) wild type (WT) strain was obtained from the National Research Council Canada Culture Collection (Ottawa, Canada). *S. stipitis* strains GS301and GS302 were derived by UV mutagenesis of the WT followed by genome shuffling of the mutants selected for improved tolerance to HW SSL (Bajwa et al., 2009, 2010).

S. stipitis WT and mutant strains were maintained individually on potato dextrose agar (PDA) plates at 4 °C and subcultured at monthly intervals. The strains were lyophilized and also stored in 20% (w/v) glycerol for long term preservation. For inoculum preparation, a loopful of cells from an isolated colony on PDA agar plate was aseptically transferred to 20 mL of liquid broth containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids supplemented with 2% (w/v) xylose in a 125-mL Erlenmeyer flask. The culture was grown with shaking (180 rpm) at room temperature (23 ± 1 °C) for 48 h (Bajwa et al., 2010).

2.2. Chemicals and substrate pretreatment

All chemicals were purchased from Sigma–Aldrich (Oakville, Canada). Two different steam-pretreated poplar hydrolysates were supplied by Mascoma Canada (formerly SunOpta BioProcess Inc., Norval, Ontario, Canada) and Forest Products Biotechnology Laboratory, University of British Columbia (UBC) (Vancouver, BC, Canada). The hydrolysate provided by Mascoma Canada was generated through a 2-stage continuous steam explosion pretreatment of poplar fibers at 200 °C for 8 min followed by hydrolysis with a cocktail of hydrolytic enzymes. The hydrolysate from UBC was produced by steam pretreatment at 170 °C for 20 min followed by impregnation in 10% SO_2. The third hydrolysate tested was produced from a mixture of maple (65%), aspen (15%), and birch (20%) wood chips collected from Eastern Canada. It was prepared by FPInnovations – Pulp and Paper Division (Pointe Claire, Québec, Canada). The prehydrolysis was done with 0.5% (w/v) SO_2 solution at 140 °C for 60 min with a 50-min ramp to temperature. The chemical composition and initial pH of each wood hydrolysate are shown in Table 1. All the hydrolysates were stored at 2–4 °C until use.

Prior to use, the pH of each hydrolysate was raised to 5.5 with 10 M NaOH. The hydrolysate was then boiled for 5 min in a microwave oven followed by gradual cooling to room temperature as done before for HW SSL (Bajwa et al., 2009).

2.3. Growth assessment of the genome shuffled strains in wood hydrolysates

The WT and genome shuffled strains, GS301 and GS302 were assessed for growth in the different wood hydrolysates using low cell density inocula. One milliliter of the inoculum culture was transferred to 50 mL of the hydrolysate in 250-mL Erlenmeyer flask. The flasks were incubated with shaking (180 rpm) at 28 ± 1 °C. Growth was monitored periodically by withdrawing samples and spreading serially diluted samples on PDA plates and counting colony forming units (CFU) after 48 h (Bajwa et al., 2009, 2010).

Table 1
Composition of various wood hydrolysates used in the study.

	Wood hydrolysates (% w/v)		
	Mixed hardwood prehydrolysate from FPInnovations	Poplar hydrolysate from Mascoma Canada	Poplar hydrolysate from UBC
Glucose	0.20	3.3	0.33
Mannose	0.4	0.12	0.2
Galactose	0.14	ND	0.06
Xylose	3.5	3.0	1.8
Arabinose	0.14	0.08	0.04
Cellobiose	ND[a]	0.23	ND[a]
Total Sugars	4.38	6.73	2.43
Acetic acid	0.95	0.85	0.43
Furfural	0.02	0.009	0.007
HMF	NT[b]	0.005	0.081
pH	1.08	4.8	1.6

[a] Not detected.
[b] Not tested.

2.4. Fermentation assessment of the genome shuffled strains in wood hydrolysates

The WT and genome shuffled strains were assessed individually for the ability to ferment the monomeric sugars in different wood hydrolysates using high cell density inocula (1.8–2.0 g/L dry cell weight). The use of high density inocula minimized cell growth, thereby allowing a focused assessment of the fermenting ability of the strains. The inocula for the WT and mutants were grown in a chemically defined xylose-containing medium in the same way as described before for HW SSL fermentations (Bajwa et al., 2009). After 48 h of growth, cells were harvested by centrifugation at 10,000*g* for 5 min at 4 °C. The pellet was washed twice with sterile distilled water and suspended to the pre-determined cell densities in 100 mL of each of the hydrolysates held in a 250-mL Erlenmeyer flask. Fermentation was carried out at 28 ± 1 °C and samples (2 mL) were withdrawn periodically for sugar and ethanol analysis.

2.5. Analytical methods

The fermentation samples were centrifuged at 8000*g* for 2 min in an Eppendorf centrifuge to obtain the supernatants. Residual glucose, xylose, acetic acid and ethanol in the supernatants were measured by HPLC using a Bio-Rad HPX-87H column eluted with 5 mM sulfuric acid (Lee et al., 1986). Other sugars were not measured because most of these were present only in very low amounts in the hydrolysates tested. Glycerol, formic acid and isopropanol at a concentration of 1% (w/v) served as the internal standards for sugar, acetic acid and ethanol measurements, respectively. All the experiments were conducted at least 3 times using independently grown inocula. Figures presented show the trends for one representative experiment.

3. Results and discussion

The mixed hardwoods pre-hydrolysate from FPInnovations and the steam pretreated poplar hydrolysate from UBC contained more pentoses than hexoses, while the enzymatically hydrolysed steam pretreated poplar hydrolysate from Mascoma Canada contained slightly more hexoses than pentoses (Table 1). In addition, the Mascoma hydrolysate did not contain any measurable galactose