Video Article

Techniques for the Evolution of Robust Pentose-fermenting Yeast for Bioconversion of Lignocellulose to Ethanol

Patricia J. Slininger[1], Maureen A. Shea-Andersh[1], Stephanie R. Thompson[1], Bruce S. Dien[1], Cletus P. Kurtzman[2], Leonardo Da Costa Sousa[3], Venkatesh Balan[3]

[1]Bioenergy Research Unit, National Center for Agricultural Utilization Research

[2]Mycotoxin Prevention and Applied Microbiology Research Unit, National Center for Agricultural Utilization Research

[3]Chemical Engineering and Material Science, Great Lakes Bioenergy Center, Michigan State University

Correspondence to: Patricia J. Slininger at Pat.Slininger@ars.usda.gov

URL: https://www.jove.com/video/54227
DOI: doi:10.3791/54227

Keywords: Bioengineering, Issue 116, *Scheffersomyces stipitis*, *Pichia stipitis*, diauxic lag, xylose, fermentation inhibitors, relative performance index, switch grass, corn stover, hydrolyzate, furfural, acetic acid, hydroxymethylfurfural

Date Published: 10/24/2016

Citation: Slininger, P.J., Shea-Andersh, M.A., Thompson, S.R., Dien, B.S., Kurtzman, C.P., Sousa, L.D., Balan, V. Techniques for the Evolution of Robust Pentose-fermenting Yeast for Bioconversion of Lignocellulose to Ethanol. *J. Vis. Exp.* (116), e54227, doi:10.3791/54227 (2016).

Abstract

Lignocellulosic biomass is an abundant, renewable feedstock useful for production of fuel-grade ethanol and other bio-products. Pretreatment and enzyme saccharification processes release sugars that can be fermented by yeast. Traditional industrial yeasts do not ferment xylose (comprising up to 40% of plant sugars) and are not able to function in concentrated hydrolyzates. Concentrated hydrolyzates are needed to support economical ethanol recovery, but they are laden with toxic byproducts generated during pretreatment. While detoxification methods can render hydrolyzates fermentable, they are costly and generate waste disposal liabilities. Here, adaptive evolution and isolation techniques are described and demonstrated to yield derivatives of the native *Scheffersomyces stipitis* strain NRRL Y-7124 that are able to efficiently convert hydrolyzates to economically recoverable ethanol despite adverse culture conditions. Improved individuals are enriched in an evolving population using multiple selection pressures reliant on natural genetic diversity of the *S. stipitis* population and mutations induced by exposures to two diverse hydrolyzates, ethanol or UV radiation. Final evolution cultures are dilution plated to harvest predominant isolates, while intermediate populations, frozen in glycerol at various stages of evolution, are enriched on selective media using appropriate stress gradients to recover most promising isolates through dilution plating. Isolates are screened on various hydrolyzate types and ranked using a novel procedure involving dimensionless relative performance index (RPI) transformations of the xylose uptake rate and ethanol yield data. Using the RPI statistical parameter, an overall relative performance average is calculated to rank isolates based on multiple factors, including culture conditions (varying in nutrients and inhibitors) and kinetic characteristics. Through application of these techniques, derivatives of the parent strain had the following improved features in enzyme saccharified hydrolyzates at pH 5-6: reduced initial lag phase preceding growth, reduced diauxic lag during glucose-xylose transition, significantly enhanced fermentation rates, improved ethanol tolerance and accumulation to 40 g/L.

Video Link

The video component of this article can be found at https://www.jove.com/video/54227/

Introduction

An estimated annual 1.3 billion dry tons of lignocellulosic biomass could support ethanol production and allow the U.S. to reduce its petroleum consumption by 30%.[1] Although plant biomass hydrolysis yields sugar mixtures rich in glucose and xylose, fermentation inhibitors are generated by the chemical pretreatment necessary to break down hemicellulose and expose cellulose for enzymatic attack. Acetic acid, furfural, and hydroxymethylfurfural (HMF) are thought to be key components among many inhibitors that form during pretreatment. In order to move the lignocellulosic ethanol industry forward, research and procedures to allow the evolution of yeast strains capable of surviving and efficiently functioning to use both hexose and pentose sugars in the presence of such inhibitory compounds are needed. A significant additional weakness of traditional industrial yeast strains, such as *Saccharomyces cerevisiae*, is the inability to efficiently ferment the xylose available in hydrolyzates of plant biomass.

Pichia stipitis type strain NRRL Y-7124 (CBS 5773), recently renamed *Scheffersomyces stipitis,* is a native pentose fermenting yeast that is well known to ferment xylose to ethanol.[2,3] The evolution of strain NRRL Y-7124 was pursued here because it has been documented to have the greatest potential of native yeast strains to accumulate economically recoverable ethanol exceeding 40 g/L with little xylitol byproduct.[4,5,6] In optimal media, *S. stipitis* strain NRRL Y-7124 produces 70 g/L ethanol in 40 hr (1.75 g/L/hr) at a yield of 0.41 ± 0.06 g/g in high cell density cultures (6 g/L cells).[7,8] Resistance to fermentation inhibitors ethanol, furfural, and HMF has also been reported,[9] and *S. stipitis* has been ranked among most promising native pentose-fermenting yeasts available for commercial scale ethanol production from lignocellulose.[10] Our objective was to apply diverse undetoxified lignocellulosic hydrolyzates and ethanol selection pressures to force evolution toward a more robust derivative of strain NRRL Y-7124 suitable for industrial applications. Key among improved features sought were faster sugar uptake rates in concentrated hydrolyzates, reduced diauxy for more efficient mixed sugar utilization, and higher tolerances of ethanol and inhibitors. The application of *S.*

stipitis to undetoxified hydrolyzates was a key focus of the research to eliminate the added operating expense associated with hydrolyzate detoxification processes, such as overliming.

Two industrially promising hydrolyzates were applied to force evolution: enzyme saccharified ammonia fiber expansion-pretreated corn stover hydrolyzate (AFEX CSH) and dilute acid-pretreated switchgrass hydrolyzate liquor (PSGHL).[11,12] AFEX pretreatment technology is being developed to minimize the production of fermentation inhibitors, while dilute acid pretreatment represents the current lowest cost technology most commonly practiced to expose cellulosic biomass for enzymatic saccharification. PSGHL is separable from the cellulose remaining after pretreatment and is characteristically rich in xylose from the hydrolyzed hemicellulose, but low in glucose. AFEX CSH and PSGHL compositions differ from one another in key aspects which were exploited to manage the evolution process. AFEX CSH is lower in furan aldehydes and acetic acid inhibitors but higher in amino acids and ammonia nitrogen sources compared with PSGHL (**Table 1**). PSGHL presents the additional challenge of xylose being the predominant sugar available. Thus PSGHL is appropriate to specifically enrich for improved xylose utilization in hydrolyzates, a weakness preventing commercial use of available yeast. Even among native pentose fermenting yeasts, the reliance on the suboptimal sugar xylose to support cell growth and repair becomes even more challenging in hydrolyzates because of a variety of reasons: nutrient deficiencies, inhibitors causing widespread damage to cell structural integrity, and disruption to metabolism due to redox imbalances.[9] Nitrogen supplementation, especially in the form of amino acids, can represent a significant operating cost for fermentations. The impact of nitrogen supplementation on isolate screening and ranking was explored with switchgrass hydrolyzates.

Improved individuals were enriched in an evolving population using multiple selection pressures reliant on natural genetic diversity of the *S. stipitis* population and mutations induced by exposures to two diverse hydrolyzates, ethanol or UV radiation. Selection pressures were applied in parallel and in series to explore the evolution progress of *S. stipitis* toward desired derivatives able to grow and ferment efficiently in hydrolyzates (**Figure 1**). The repetitive culturing of functional populations in increasingly challenging hydrolyzates was accomplished in microplates employing a dilution series of either 12% glucan AFEX CSH or else PGSHL prepared at 20% solids loading. The application of ethanol-challenged growth on xylose in continuous culture further improved AFEX CSH adapted populations by enriching for phenotypes demonstrating less susceptibility to ethanol repression of xylose utilization. The latter feature was recently shown problematic to pentose utilization by strain NRRL Y-7124 following glucose fermentation.[8] Enrichment on PSGHL was next explored to broaden hydrolyzate functionality.

Putative improved derivatives of *S. stipitis* NRRL Y-7124 were isolated from each phase of the evolution process using targeted enrichment under stress conditions and dilution plating to pick colonies from the most prevalent populations. Dimensionless relative performance indices (RPIs) were used to rank strains based on overall performance, where kinetic behavior was evaluated on the different hydrolyzate types and nutrient supplements applied. Although the successes of various adaptation procedures to improve the functionality of *S. stipitis* in lignocellulosic hydrolyzates have been previously documented, strains demonstrating economical ethanol production on undetoxified hydrolyzates have not been previously reported.[13-17] Using the evolution procedures to be visualized in more detail here, Slininger *et al.*[18] developed strains that are significantly improved over the parent strain NRRL Y-7124 and are able to produce >40 g/L ethanol in AFEX CSH and enzyme saccharified switchgrass hydrolyzate (SGH) appropriately supplemented with nitrogen sources. These novel strains are of future interest to the developing lignocellulose to ethanol industry and as subjects of additional genomics studies building on those of previously sequenced strain NRRL Y-11545.[19] A genomics study of top strains produced during various phases of evolution diagramed in **Figure 1** would elucidate the history of genetic changes that occurred during development as a prelude to further strain improvement research.

Protocol

1. Prepare Starting Materials and Equipment for Assays

1. Prepare hydrolyzates using 18 to 20% initial biomass dry weight in the pretreatment reaction for use in the evolution, isolation and ranking procedures. See Slininger *et al.* 2015[18] for the detailed methods to prepare AFEX CSH, PSGHL, and SGH with nitrogen supplements N1 or N2 used in evolution, isolation or ranking. See **Table 1** for composition of each hydrolyzate type.
   NOTE: Nitrogen fortifications of SGH were designated as SGH-N1 or SGH-N2 defined as follows: SGH-N1 = SGH fortified to 42:1 molar carbon to nitrogen ratio (C:N) with nitrogen sources including urea, vitamin-free amino acids from casein, D,L-tryptophan, L-cysteine, and vitamins from defined sources (such that ~15% of molar nitrogen N is primary amino nitrogen (PAN) and ~85% of N is from urea); SGH-N2 = SGH fortified to 37:1 C:N with urea and soy flour as the lowest cost commercial source of amino acids and vitamins, providing ~12% of N from PAN and 88% as urea.
2. Acquire a lyophilized culture of the parent strain, *Scheffersomyces stipitis* NRRL Y-7124 (CBS 5773) from the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, Illinois), and prepare glycerol stock cultures as directed. Maintain stock cultures of the parent yeast and its derivatives in 10% glycerol at -80 °C.
   1. Streak glycerol stocks to yeast malt peptone dextrose (YM) agar plates (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, 20 g/L agar) and incubate 48-72 hr at 25 °C.[8] Developed plates can be stored up to a week at 4 °C prior to use as liquid pre-culture inocula.
3. Prepare Optimal Defined Medium (ODM), a synthetic medium compatible with industrial formulation procedures[20] that was previously developed for optimal conversion of xylose to ethanol by the parent strain *Scheffersomyces (Pichia) stipitis* NRRL Y-7124.[7] Use the defined medium in all precultures and cultures for fermentation performance bioassays and also for ethanol challenged, xylose-fed continuous culture evolution. ODM composition is listed for reference in **Table 2**.
4. As described previously,[18] analyze cell biomass using a cuvette- or micro-plate reading spectrophotometer, as appropriate, to measure culture absorbance at 620 nm (A_{620}). Quantitate sugars, ethanol, furfural, hydroxymethylfurfural (HMF), and acetic acid in culture samples using high performance liquid chromatography (HPLC) or robotic enzymatic determination of glucose and xylose for higher throughput.

2. Enrich Robust Derivatives during Serial Transfer on AFEX CSH

1. Inoculate a preculture of strain NRRL Y-7124. Transfer cells from YM agar to 75 ml ODM + 150 g/L xylose to maintain the ability to grow under osmotic stress. Incubate precultures in 125 ml flasks closed with silicon sponge plugs 24 hr at 25 °C with shaking (150 rpm, 1" orbit).
2. Thaw frozen aliquots of 6-12% glucan AFEX CSH in cold water for use. Adjust pH to 5 if needed and filter sterilize prior to preparing a dilution series in 96-well microplates.
3. Fill microplates with 50 µl per well and 8 wells per hydrolyzate dilution, then inoculate with a few microliters of preculture per well to allow an initial absorbance (620 nm) A_{620} ≥0.1. Incubate plates statically 24-48 hr at 25 °C in a plastic box with a wet paper towel for humidity.
4. Using the most concentrated hydrolyzate dilution that visibly grew to A_{620} >1, transfer 1-5 µl to each well of a new hydrolyzate dilution series to achieve initial A_{620} ≥0.1.
5. Monitor growth culture A_{620} in microplates with a spectrophotometer. An uninoculated dilution series serves as a control and blank. Plate lids are left on during monitoring to prevent contamination, and the readings adjusted accordingly.
6. Prepare glycerol stocks of adaptation cultures regularly for subsequent isolation of improved strains or for use in reinoculating the continuing hydrolyzate dilution series. To prepare stocks, pool four wells (200 µl) of the greatest hydrolyzate concentration colonized. Mix 1:1 with 20% sterile glycerol in cryovials, to freeze cells in 10% glycerol at -80 °C.
7. Repeat steps 2.3-2.6 until growth in 12% glucan AFEX CSH is consistently visible at 24 hr.

3. Isolate Single Cell Tolerant Derivatives after Enrichment on AFEX CSH

1. Streak selected glycerol stocks of adaptation cultures to YM agar, and use streaks to inoculate 50 µl of 3% or 6% glucan AFEX CSH (pH 5) in each of three microplate wells (initial A_{620} ~0.1). Incubate microplates 24 hr as in step 2.3. Pool replicate colonized wells at the highest hydrolyzate strength with strong growth, and dilution plate to YM or 6% glucan AFEX CSH agar. Prepare the latter as a 1:1 mix of filter sterilized 12% glucan AFEX CSH with warm autoclaved 30 g/L agar solution.
2. Pick 5 single colonies from the highest dilution plate showing growth after 24-48 hr incubation at 25 °C to freeze as glycerol stock cultures. Streak each colony to a YM plate. Incubate 24 hr. With a sterile loop, transfer a developed streak of cells to a small volume of 10% glycerol, mix to suspend cells, and distribute to 2-3 cryovials for freezing at -80 °C.

4. Evaluate Performance of AFEX CSH Tolerant Derivatives Compared to Parent

1. Test Isolates in Simple 6% Glucan AFEX CSH Batch Cultures.
    1. Transfer cells from 48 hr plates streaked from glycerol stocks to pH 7 potassium phosphate buffer (0.4 mM) to prepare cell suspensions with A_{620} = 10. Use 1 µl of each suspension to inoculate each of four wells of 50 µl 3% glucan hydrolyzate to initial A_{620} = 0.2. Incubate microplates 24 hr as in step 2.3. Prepare the 3% glucan AFEX CSH at pH 5 by diluting 12% glucan AFEX CSH 1:3 with sterile water.
    2. Transfer two 24 hr microplate wells to inoculate 25 ml precultures of pH 5 12% glucan AFEX CSH used at 50% of full strength. Incubate precultures in 50 ml flasks with silicon sponge closures for 24 hr at 25 °C, ~150 rpm (1" orbit). Prepare the half-strength 12% glucan AFEX CSH by diluting the hydrolyzate 1:1 with sterile water.
    3. Use half-strength hydrolyzate precultures to inoculate similar 25 ml growth cultures to initial A_{620} = 0.1. Incubate the growth cultures as above (4.1.2) and sample daily (0.2 ml). Monitor accumulations of biomass (A_{620}) and concentrations of sugars and fermentation products (HPLC).
2. Alternatively, repitch (*i.e.*, harvest and reuse) populations of 6% glucan AFEX CSH-grown yeast for testing in 8% glucan hydrolyzate batch cultures, as described previously.[18]
3. Alternatively, further test populations of repitched 6% glucan AFEX CSH cultures by feeding with 12% glucan hydrolyzate, as described previously.[18]
4. Test diauxic lag of isolates in batch cultures of ODM with mixed sugars.
    1. Inoculate precultures of 75 ml ODM with 150 g/L xylose in 125 ml flasks with silicone sponge closures by loop transfer from YM glycerol streaks. Incubate 24 hr as in step 2.1.
    2. Use precultures to inoculate similar 75 ml test cultures with ODM containing 75 g/L of glucose and 75 g/L of xylose at pH 6.5. Shake flasks at 25 °C, 150 rpm. Sample daily.
    3. Alternatively, test the impact of 0-15 g/L acetic acid on diauxy during fermentation of glucose and xylose by using a similar protocol, except initial pH is set at 6.0 ± 0.2 in test cultures to maintain pH 6-7 during acetic acid consumption, as previously described.[18]

5. Apply Continuous Culture to Select for Ethanol-challenged Xylose Utilization

1. Prepare ODM as the continuous culture feed medium with 60-100 g/L xylose, 20-50 g/L ethanol at pH 6.3 ± 0.2. Choose combinations of xylose and ethanol to simulate the partial fermentation of 150 g/L xylose, assuming a potential yield of ~0.5 g ethanol/g xylose, and to accommodate the potential for 50-70 g/L ethanol to occur.[8]
2. To prepare the continuous culture inoculum, transfer a representative AFEX CSH-tolerant colony (analogous to Colony 5 in example, **Figure 1**) by loop from a 48 hr YM plate to 75 ml of the ethanol-free ODM (100 g/L xylose, in this case) in 125 ml flasks. Incubate at 25 °C, 150 rpm (1" orbit) for 24 hr. Choose the preculture ODM xylose concentration to match that of the initial continuous culture holding volume.
3. Inoculate a continuous culture holding volume of ODM at pH 6.3 ± 0.2 having 100 g/L xylose + 20 g/L ethanol with ~5-10 ml of a preculture concentrate of a AFEX CSH-tolerant isolate (analogous to Colony 5, **Figure 1**) to obtain 100 ml at initial A_{620} ~0.5. Maintain the culture holding volume (V_R), at 25 °C in a jacketed 100 ml spinner flask stirred at 200 rpm and outfitted with sterilizable pH electrode, pH controller and temperature-controlled circulating water bath.
4. Initially, since cell growth will be slow due to the ethanol exposure, set up the feed medium to dose using a pH-actuated pump. Set the pump to feed pH-6.3 ODM with 100 g/L xylose and 50 g/L ethanol when the culture fermentation drops the pH to 5.4, thus stopping further pH drop.

Maintain the volume at 100 ml with an effluent pump continuously skimming the culture surface. Consequently, ethanol concentration rises with continued metabolism and feeding.

5. Measure effluent and draw samples (1-2 ml) from continuous cultures every 48-72 hr for analysis of viable cell concentration, products and substrates, as previously described.[18] To find viable cell concentration, make serial 1:10 dilutions of samples in buffer (see 4.1.1) and spread plate the dilutions to YM agar (see 1.2.1). Based on effluent collection rates (Q), calculate the dilution rate (D) (Q/V_R), and, in the example of *S. stipitis,* it varied from 0 to 0.01 hr^{-1}.
6. Save glycerol stocks regularly by isolating from viability plates of buffered sample dilutions forming 30-100 colonies, representing most prevalent robust colonists at that time in enrichment. Flood plates with ~5 ml of 10% glycerol to allow preparation of duplicate cryovials. For maintenance or a respite, stop the continuous culture and restart as needed using most current (resistant) glycerol stock(s).
7. To restart, streak the glycerol stock(s) of most resistant populations to YM agar and transfer 24-48 hr cells by loop to a pre-culture of ethanol-free ODM for incubation as above. Inoculate the 100 ml holding volume of ODM to initial A_{620} 0.5 using a concentrate of the precultured yeast, and allow the culture to grow batch-wise to stationary phase before the feed medium flow is restarted.
8. If cultures can grow steadily at >0.01 hr^{-1} on xylose in the presence of >25 g/L ethanol, start the continuous culture feed (ODM + 60 g/L xylose + ethanol ranging 30 to 50 g/L) at a low dilution rate ~0.01 hr^{-1} to the 100 ml holding volume (initially ODM + 60 g/L xylose + 20 g/L ethanol). Over time, raise the ethanol in the feed toward 50 g/L. Capture advanced populations in glycerol stocks.
   NOTE: Maintaining the low dilution rate allows formation of a high enough cell concentration to reduce oxygen availability and support fermentation.
9. Optionally, apply ultra-violet (UV) irradiation monthly to glycerol stock populations used to reinoculate continuous cultures.
   1. Resuspend colonies from glycerol stock streaks in 10 ml of ODM (as in precultures) with 60 g/L xylose, and pool all stocks in a single sterile flask. Apply the pooled cell suspension at ~5 x 10^8 viable cells/ml to just cover the bottoms of 4-5 Petri plates with 6 ml/plate. To obtain 6 ml coverage of a standard disposable petri plate, dispense 15 ml to overcome surface tension, and then remove 9 ml.
   2. Expose each open culture plate to UV from the light source in a biological safety cabinet. Adjust the UV exposure time or distance to obtain the desired kill rate >90%. Here, expose for 45 min at around 56 cm from the source.
   3. Dilute plate cell suspensions before and after irradiation. Estimate kill rate based on colony forming units. For the *S. stipitis* example, the kill rate was ~97%.
   4. Transfer the UV-exposed cultures (24-30 ml) to a foil-covered 50 ml flask, and incubate at 25 °C and 150 rpm for 24 hr to allow the small percentage of viable cells to repopulate to ~1 x 10^8 viable *S. stipitis* cells/ml. By preventing photo reactivations, the foil cover preserves mutations while cultures are incubated.
   5. Use the repopulated mutagenized culture to inoculate continuous cultures to ~1 x 10^7 viable cells/ml. Restart the ethanol-enriched ODM + 60 g/L xylose medium feed to continue the selection process.

6. Evaluate Glycerol Stock Populations and Identify Those with Improved Xylose Fermentation in the Presence of Ethanol

1. Streak selected glycerol stock cultures to YM agar as the first step in the screen for best growth and fermentation of xylose in the presence of ethanol.
2. Transfer each evolved population to be screened from agar streaks to 75 ml precultures in ODM with 150 g/L glucose, and incubate in 125 ml flasks 96 hr prior to use as inocula for test cultures. The large glucose-grown populations will require induction of enzymes for xylose utilization.
   1. To test induction, harvest each culture, suspend cells to 30 ml, initial A_{620} of 40 in ODM +60 g/L xylose + 30-45 g/L ethanol, and incubate at 25 °C, 150 rpm (1" orbit) in 50 ml flasks with silicon sponge closures. Draw samples twice daily for monitoring xylose uptake by HPLC analysis.
3. Alternatively, as described in Slininger *et al.*,[18] to assess growth on xylose in the presence of ethanol, prepare precultures of each evolved population by loop transfer to 75 ml of ODM with 150 g/L xylose in 125 ml flasks, and incubate 96 hr at 25 °C, 150 rpm (1" orbit). Inoculate test cultures to a low initial A_{620} of 0.1 in 25 ml of ODM + 60 g/L xylose + 30-45 g/L ethanol in 125 ml flasks. Incubate flasks at 25 °C, 300 rpm, 1" orbit and sample.

7. Isolate Single-cell Colonies That Utilize Xylose in PSGHL When Ethanol Is Present

1. Based on results of step 6, select glycerol stock populations showing superior ability to grow on and ferment xylose in the presence of ethanol. Use these to streak plates. The streak plates are used to prepare dense, buffered cell suspensions of A_{620} = 5. Then cell suspensions are used to inoculate precultures in 96-deep well plates to A_{620} = 0.5. Inoculate 1 ml precultures on PSGHL mixed 1:1 with ODM + 50 g/L xylose (no ethanol). Incubate precultures in 96-well, deep well plates with vented low evaporation covers for 48 hr at 25 °C, 400 rpm, 1" orbit. Separate different glycerol stock populations by empty wells.
2. Using each preculture, inoculate sixteen 1 ml replicate cultures to initial A_{620} ~0.5 in 1:1 PSGHL:ODM+50 g/L xylose with 20, 30, or 40 g/L ethanol to enrich tolerant colonists. Incubate enrichment cultures similarly to precultures.
3. For each different glycerol stock, harvest the culture wells with highest ethanol concentration allowing growth and xylose use. Plate each cell line to YM agar to obtain prevalent single colonies to preserve in glycerol. Pick ten colonies per cell line and streak each to a YM agar plate for glycerol stock preparation.

8. Further Enrich Robust Evolved Strains during Serial Transfer on PSGHL, as for AFEX CSH

1. Prepare pre-cultures of NRRL Y-7124 (parent), AFEX CSH-tolerant evolved isolate, and continuous culture evolved population with enhanced ability to use xylose in the presence of ethanol. Inoculate 75 ml of ODM + 150 g/L xylose by loop from glycerol stock streaks as described above (step 2.1) for the AFEX CSH hydrolyzate adaptation process.
2. Dilute PSGHL with water to provide a series of increasing concentrations. Prepare a 96-well micro-plate for each of the three cell lines by using each PSGHL dilution to fill 8 wells with 50 µl. Use preculture to inoculate each 50 µl micro-culture of the dilution series to initial A_{620} ~0.1-0.5.
    1. Continue the evolution of yeast in increasing strengths of PSGHL using the same procedure as the AFEX CSH hydrolyzate adaptation detailed above (step 2) until cultures are able to grow in the full strength hydrolyzate at a stable 14-d average ratio of ΔA_{620} per Δtime as serial transfers are continued an additional four months.

9. Isolate Single-cell Colonies Using PSGHL Gradients with or without Ethanol Challenge

1. Obtain single-cell isolates directly from full strength PSGHL final adaptation plates by dilution plating to YM agar. Pick ten large colonies for each of the three cell lines and bar streak to YM plates to prepare glycerol stocks as previously described (step 3.2).
2. Optionally, explore earlier time points in the evolution process by isolating from stored glycerol stocks of the three cell lines.
    1. Inoculate a preculture for each cell line by loop from glycerol stock streaks and incubate 24 hr on 30 ml ODM + 50 g/L xylose (50 ml flasks, 25 °C, 150 rpm).
    2. Challenge cells from precultures to growth in hydrolyzate by inoculating 50 µl of 50% PSGHL in microplate wells to an initial A_{620} ~0.2 and incubating statically 48 hr.
    3. Spread 0.1 ml of each enriched culture onto PSGHL gradient agar plates ranging from 0 to 50% strength hydrolyzate (delivering ~300-400 viable cells per plate), and pick 10 single colonies per cell line from the highest possible hydrolyzate concentration area of the gradient.[21] Streak picked colonies to YM for glycerol stock preparation as in step 3.2.
    4. As an alternative to step 9.2.3, instead of inoculating the gradient agar plates, inoculate wells of 96-well micro-plates to initial A_{620} ~0.2, where the wells are designed to contain a range of hydrolyzate concentrations from 50 to 100% strength and ethanol from 10 to 40 g/L. In this case, develop micro-plates 72-96 hr and otherwise as in step 2.3. Isolate ten single colonies from wells of the harshest hydrolyzate-ethanol combinations showing growth via dilution plating, and prepare glycerol stocks as in step 3.2.

10. In a Primary Screen, Eliminate Inferior Isolates by Comparing and Ranking Performances on PSGHL at Two Nutrient Conditions

1. Apply a high throughput deep well plate screen of PSGHL performance to screen five sets of thirty isolates along with NRRL Y-7124 parent and AFEX CSH-tolerant isolate (analogous to Colony 5 of the **Figure 1** evolution example) as controls to choose six top strains (20%) from each set to pass to the secondary screening level (step 12).
2. Carry out the screen in deep well plates filled 1 ml per well and covered with stainless steel lids with black silicone low evaporative seals. Clamp all plates to the board of the incubator/shaker and operate at 25 °C and 400 rpm (1" shaker orbit). Design all deep well plate filling patterns to allow separation of different isolates by open wells.
3. To begin cultivations, pick a bead of cells from glycerol streaks prepared from 30 isolates obtained as above (in steps 3, 7, and 9) to duplicate wells of ODM + 50 g/L xylose and incubate 48 hr.
4. As a challenge medium for preculturing isolates, prepare 50% PSGHL by mixing PSGHL 1:1 with ODM + 10 g/L glucose + 50 g/L xylose. For screening 30 isolates and two control strains, 2 plates with 2 wells per each of 16 isolates are filled, leaving empty wells between the different isolates.
5. For challenge cultures, transfer, a 50 µl volume of ODM pre-cultures (A_{620} ~10) to each of two 50% PSGHL challenge culture wells for each of the isolates and controls to obtain an initial A_{620} ~0.5 and incubate 72 hr.
6. Use two test media of different nutritional richness for screening isolates: 60% PSGHL + ODM nutrients; and 75% PSGHL + ODM + YM nutrients. Add ODM nutrients (excluding sugars) at half of the strength as standard for ODM use with 50 g/L sugar (step 1.3). As designated, add YM nutrients (excluding sugars) at half of the standard strength of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone.
7. Inoculate test cultures by transferring 50 µl of 72 hr 50% PSGHL challenge pre-cultures to inoculate 1,000 µl to initial A_{620} ~0.5 in five deep wells for each of two test media.
8. Sample each isolate daily. Pipette the contents of a well to a microfuge tube and centrifuge (5,533 x g, 15 min) to obtain supernate for ethanol, glucose and xylose high throughput analyses. Measure biomass as A_{620} in 96-well micro-plates (200 µl/well) with a spectrophotometer.
9. Within each set of 30 isolates tested (5 sets for *S. stipitis* NRRL Y-7124 evolved strains), calculate relative performance indexes (RPI) as described in step 11 below and use to rank each strain based on ethanol yield (maximum ethanol accumulated per initial sugar supplied) and xylose uptake rate (xylose concentration consumed per initial 96 hr) on both test media.

11. Rank Isolates in the Primary PSGHL Screen Using Relative Performance Index (RPI)

1. Following primary screening, calculate dimensionless relative performance indices (RPI) in order to rank the 30 isolates in each of the five different experiments to screen isolates on PSGHL with two different nutrient formulations per experiment, *i.e.,* 60% PSGHL and 75% PSGHL.

2. Calculate the statistical parameter F for use in ranking isolate performance within a group of isolates tested in a given experiment: $F = (X-X_{avg})/s$. Here, X is the performance parameter, such as yield (Y) or rate (R), observed for each individual isolate, while X_{avg} and s are the average and standard deviation, respectively, of X observed for all isolates within a given experiment. For normal distributions, $-2 < F < 2$.
3. For each isolate in an experiment, calculate relative performance based on rate, $RPI_R = (2+F_R) \times 100/4$, and similarly calculate relative performance based on yield, $RPI_Y = (2+F_Y) \times 100/4$. The value of RPI ranges from ~0 to 100 percentile (lowest to highest). Then calculate RPI averages for each isolate within a given hydrolyzate experiment as follows: $RPI_{avg} = (RPI_Y + RPI_R)/2$, where yield and rate contributions are given equal weighting in this application.
   Note that in general, yield and rate parameters can be weighted if deemed unequal in importance.
4. Compute $RPI_{overall}$ across the n types of hydrolyzates tested: $RPI_{overall} = \Sigma_{i=1,n}[(RPI_Y + RPI_R)/2]_i/n$. Consider each isolate in an experimental set of 30 isolates and 2 controls. During primary ranking of the ~150 single-colony isolates adapted to xylose-rich PSGHL, the $RPI_{overall}$ is calculated for rates and yields across the two PSGHL formulations applied in the screen, *i.e.,* 60% PSGHL and 75% PSGHL, where n = 2.
5. Based on $RPI_{overall}$ within each experiment, choose the top percentage of isolates to pass to the secondary screen. In this example, the top 20% of strains are chosen to pass through (*i.e.,* 30 of 150 tested).

12. In a Secondary Screen, Compare Top Primary Screen Performers on Multiple Complete Hydrolyzates (>100 g/L Mixed Sugars) to Reveal Highest Functioning Robust Strains

1. Compare top PSGHL performers on 6% glucan AFEX CSH and SGH amended with two levels of nitrogen, SGH-N1 and SGH-N2 (compositions as in **Table 1**) for final ranking. Screen the 30 isolates that were the top performers in the primary deep well plate screen of PSGHL (steps 10 and 11) and the two controls (parent strain NRRL Y-7124 and AFEX CSH-tolerant isolate (analogous to Colony 5, **Figure 1** example).
2. Begin cultivations by picking a bead of cells from glycerol streaks to duplicate deep wells of 1 ml ODM + 50 g/L xylose as before and incubate 48 hr in the deep well plate system (step 10.2). Then transfer 50 µl of ODM precultures to 50% SGH challenge cultures, and incubate in the deep well plate system for 72 hr to obtain A_{620} at ~10). Prepare the 50% SGH by mixing SGH 1:1 with sugarless ODM + 50 g/L xylose (pH 5.6).
3. For each of the isolates, inoculate a 16 ml aliquot of SGH-N1 or SGH-N2 with the cell pellet (15 min, 2,711 x g) from three wells of challenge culture to yield initial test culture A_{620} ~2.0. Incubate test cultures at 25 °C, 180 rpm (1" orbit) in 25 ml flasks with silicone sponge closures. Sample flasks daily and analyze per the PSGHL screen.
4. Similarly, screen isolates as in steps 12.2 and 12.3, but this time substitute SGH-N1 and SGH-N2 with 6% glucan AFEX CSH at pH 5.2 for use in preparing the challenge culture medium and test culture medium. For the 50% strength challenge cultures, use 6% AFEX CSH diluted 1:1 with water since it is nitrogen sufficient without amendment.
5. Repeat steps 12.1-12.4 to duplicate results.

13. Rank the Performances of Isolates in the Secondary Screen Using $RPI_{overall}$ to Rate Use of Multiple Complete Hydrolyzates

1. Calculate relative performance indices (RPI) in order to rank each of the top 20% of isolates (~30 out of 150) from the primary screen that have been tested in the secondary screen on enzyme saccharified hydrolyzate formulations of varying nutritional richness.
2. Score each isolate tested in the secondary screen based on xylose uptake rate and ethanol yield performed on three enzyme-saccharified pretreated hydrolyzate formulations run in duplicate, including AFEX CSH (i = 1 and 2), SGH-N1 (i = 3 and 4) and SGH-N2 (i = 5 and 6).
3. Calculate the $RPI_{overall}$ for each isolate, and apply this ranking parameter to further winnow the list of superior isolates: $RPI_{overall} = \Sigma_{i=1,n}[(RPI_Y + RPI_R)/2]_i/n$, where n = 6.
   NOTE: Demonstrate kinetics of superior isolates in SGH-N2 hydrolyzates in flask cultures by following the procedures in Slininger *et al.*[18]

Representative Results

S. stipitis was evolved using combinations of three selection cultures, which included AFEX CSH, PSGHL, and ethanol-challenged xylose-fed continuous culture. **Figure 1** shows the schematic diagram of the evolution experiments performed along with the isolates found either to perform most effectively overall, or most effectively on one of the hydrolyzates tested. **Table 3** shows the NRRL accession numbers of these superior isolates and summarizes the adaptation stresses applied in the process of achieving the enriched population from which each strain was isolated. Some isolates were seen to have superior relative performance on one or two hydrolyzate types, but 7 of 11 isolates performed well on all of the hydrolyzate types, even though most of these were exposed and challenged by only a single type during evolution. The successes of the various evolution approaches taken here are demonstrated in **Figures 2**-**7** and **Table 4**.

Figure 2 depicts the distinct improvements seen after the first phase of adaptation in which robust derivatives of strain NRRL Y-7124 were enriched during serial transfer to increasing concentrations of AFEX CSH (Step 2). In cultures growing on 6% glucan AFEX CSH, the evolved population demonstrates more rapid accumulation and higher final titers of ethanol than the parent strain. More rapid glucose utilization also is seen along with more rapid xylose uptake immediately following the depletion of glucose. Additionally in **Figure 3**, the stability of the changed population features is demonstrated in the synthetic medium ODM with a mixture of 87 g/L glucose and 66 g/L xylose. In this case, both the enriched population (**Figure 3B**) and isolated colonies (Colony 1 and Colony 5, **Figure 3C** and **3D**, respectively) are able to outperform the parent strain by using the xylose more efficiently to make ethanol more rapidly, reducing time to peak ethanol by at least 4 days. Significantly higher ethanol concentrations were accumulated by evolved strains on ODM (55-60 g/L) compared to the parent (40-45 g/L). The mutations, which led to a stable phenotype of reduced diauxy during the glucose-xylose transition and more efficient xylose fermentation, arose as the yeast population evolved in AFEX CSH.

Further improvements to Colony 5 were pursued by submitting it to natural selection in xylose-fed continuous culture in the presence of increasing levels of ethanol up to 50 g/L. Under this condition, the yeast population retained in the continuous culture must be able to induce enzymes for xylose utilization as sole carbon source in order for it to be able to grow at a rate high enough to populate the fermentor despite a steady dilution rate. In the parent strain, the induction of xylose enzymes begins to be inhibited at ethanol concentrations as low as 15-20 g/L.[8] Derivatives of Colony 5 were captured in glycerol stocks at early and late time points of operation of the continuous culture, and **Figure 4** shows the successful improvement in xylose utilization in the presence of 40 g/L ethanol observed in evolved derivatives of Colony 5 compared with the NRRL Y-7124 parent and the initial Colony 5 inoculated to the process.

Isolates arising from all phases of the adaptation scheme of **Figure 1** were screened in PSGHL to identify those with superior ability to ferment xylose (Step 10.1). The isolates shown in **Figure 5** are among the best performers on PSGHL out of ~150 ranked in this primary screen and in all secondary screens of performance as described below. To indicate improvement of evolved isolates relative to their parent, the performance of each isolate was expressed as the ratio of kinetic parameter values of isolate to parent strain. Ratio values of "one" occurred if the isolate performance was equivalent to the parent. **Figures 5a** and **5b** summarize top isolate performances on 60% and 75% strengths of PSGHL with ODM or ODM+YM nutrient supplements. As the hydrolyzate concentration and harshness was increased, the performance ratios decreased. In the 60% strength PSGHL, five of seven top isolates exposed to PSGHL selection pressure performed many times better than NRRL Y-7124 (isolate 1). Four isolates performed better than Colony 5 (isolate 33), which had evolved during exposure to AFEX CSH but had no previous selective exposure to PSGHL. However, in the 75% strength of PSGHL, only 3 isolates significantly surpassed both the parent and Colony 5 (isolate 33) despite the added nutrients. Of these, superior isolates 15 and 16 were previously challenged with increasing concentrations of PSGHL as a selection pressure guiding evolution. Isolates 15, 14 and 3 were only exposed to PSGHL, while 11, 13 and 16 had multiple exposures. Isolates 3, 11, and 13 were from earlier time points during the evolution on PSGHL and so had somewhat less opportunity to develop tolerance compared to 14, 15 and 16. While it is evident in **Figure 5** that serial transfer to increasing strengths of PSGHL served to develop strains robust to its inhibitory environment, it is also evident that exposure of the parent strain NRRL Y-7124 to AFEX CSH alone could potentially generate isolates such as Colony 5 (33) with cross tolerance to PSGHL. Thus, it was indicated that robust strains able to perform in multiple hydrolyzates could be found by enrichment of tolerance in one hydrolyzate followed by performance screening in another. Isolates obtained from the xylose-fed continuous culture were also screened on PSGHL 60% and 75% strengths with nutrients and also added glucose at 75 g/L in order to sustain the ethanol challenge and test diauxic lag on xylose following glucose utilization. While isolates 27, 28 and 30 were superior to others from this phase of adaptation, both yield and xylose uptake rate performance ratios were similar to the parent on 75% PSGHL with added ODM and YM nutrients, which is not necessarily surprising in that none had previous exposure to this hydrolyzate (see also Slininger *et al.*[18] for additional data not shown here for the cultures provided 75 g/L glucose).

The best strains selected from the primary screen on xylose-rich PSGHL were subsequently screened on three complete hydrolyzates. These hydrolyzates (**Table 1**) included AFEX CSH and dilute-acid pretreated SG treated with commercial cellulases and supplemented at two different nitrogen levels (SGH-N1 and SGH-N2) to identify the most versatile isolates with respect to variations in inhibitor and nutritional environment. The relative performance indices (RPIs) were calculated for the fermentation of each isolate within each hydrolyzate (**Figure 6A**). **Figure 6B** shows the combined overall performance indices calculated for each isolate. Five isolates (3, 14, 27, 28, 33) had overall RPI above 60, which ranked them as the most robust to all of the variations of hydrolyzate and nutrient conditions combined. Reviewing **Table 3**, both isolates 3 and 14 were evolved in PSGHL while 27, 28, and 33 were evolved in AFEX CSH or AFEX CSH and ethanol-challenged continuous culture on xylose. None of the strains exhibiting superior multiple hydrolyzate use were evolved on more than one hydrolyzate.

Some strains were "specialists" performing better on either SGH-N1/2 or AFEX CSH. Those isolates performing best as specialists on SGH hydrolyzates (11, 16 and 9) were obtained by evolution on PSGHL as the final or only challenge. For isolates 11 and 16, which were initially enriched via increasing AFEX CSH challenge, the ability to efficiently utilize AFEX CSH was not actively selected during the lengthy final enrichment in PSGHL, and key genetic factors supporting its use were evidently lost. Conversely, isolates 14, 25, 27, 30 and 33 were superior performers on AFEX CSH, and all except isolate 14 were evolved on AFEX CSH with or without ethanol challenge. So as expected, directed evolution on AFEX CSH or PSGHL tended to select for yeast well adapted to the selection hydrolyzate. The one exception in this regard was isolate 14. Isolate 14 originated from the PSGHL only challenge and was isolated from YM agar dilution plating of the final PSGHL enrichment culture. Slininger *et al.*[18] showed this isolate to have superior capability of xylose enzyme induction in glucose-grown cells in the presence of 5-15 g/L acetic acid and reduced diauxic lag on ODM with 75 g/L each of glucose and xylose, despite >30 g/L ethanol occurring prior to the glucose-xylose transition point.

The superior kinetics of evolved strains relative to the parent strain *S. stipitis* NRRL Y-7124 were demonstrated as shown in **Table 4**, representing the results of low level aeration flask cultures inoculated to initial A_{620} 8.4 ± 2.5 75 ml SGH-N2 (pH 6.2) incubated in 125 ml flasks with silicone sponge caps at 25 °C, 150 rpm (1"orbit), and in **Figure 7**, representing moderate aeration flask cultures inoculated to initial A_{620} 0.5 in 23 ml SGH-N2 per 50 ml flask.[18] These conditions represent two different types of operation suggested by the literature as being potentially commercially promising for ethanol production by *S. stipitis*.[8,22] In the first instance of low level aeration, the high cell density provides for rapid fermentation to begin immediately. Whereas in the second instance, the low cell density and higher level aeration lead to logarithmic growth to build the population and accelerate growth-associated sugar conversion to ethanol. These data demonstrate that the evolved strains have significant kinetic advantages over the parent strain: more rapid glucose uptake rate (**Table 4**), more rapid specific xylose uptake rate (**Table 4**), more rapid ethanol productivity on both glucose and xylose and overall (**Table 4**), shorter lag preceding growth (**Figure 7**), and shorter diauxic glucose to xylose transitional lag. These improvements allowed ethanol to accumulate to over 40 g/L and to peak days earlier (**Figure 7**) than was seen for the parent NRRL Y-7124. Higher overall ethanol productivity (**Table 4**) was achieved at 1.5 to 5 times that of the parent strain (**Table 4**), depending on evolved strain.

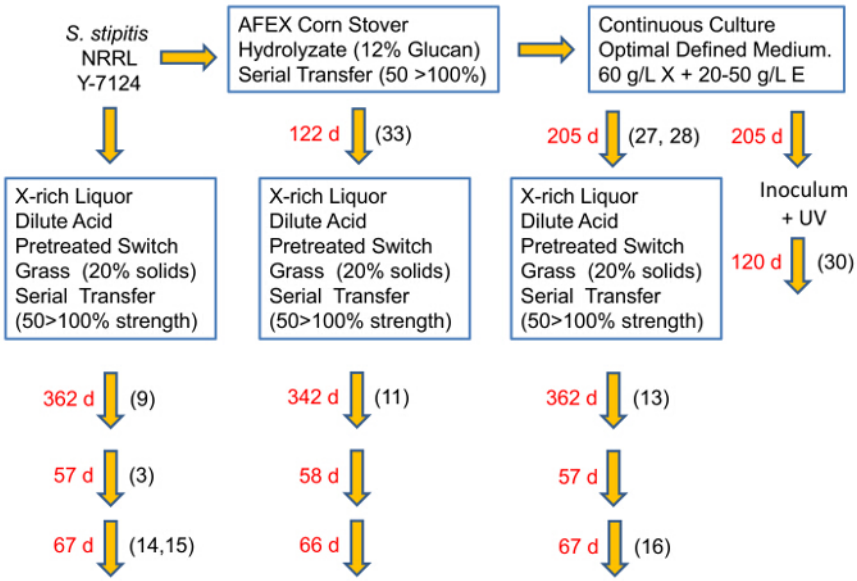

Figure 1: *Scheffersomyces stipitis* adaptation flow chart. The diagram shown indicates the order of the stresses applied during the adaptation process and the points of recovery of superior isolates (numbers in parenthesis). See also **Table 3** isolate key as reference for strain identities. To provide time orientation, the numbers in red indicate the number of days in each phase of adaptation. For the serial transfer phases in AFEX CSH and xylose-rich PSGHL, each day of adaptation represents approximately 2-4 generations. For the continuous culture phase (205 days total), the dilution rate D was variable at ~ 0-0.1 hr^{-1} during 125 d of operation with pH-actuated feeding. In the next 80 days, operation was at a continuous flow with D at 0.012 hr^{-1}, providing a generation time (ln 2)/(D) of 58 hr, or 1 generation per 2.4 d at steady state. Next a sample of the adapted population from the 205-day continuous culture was mutagenized with UV light and inoculated to a continuous culture operated with D at 0.012 hr^{-1}. (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

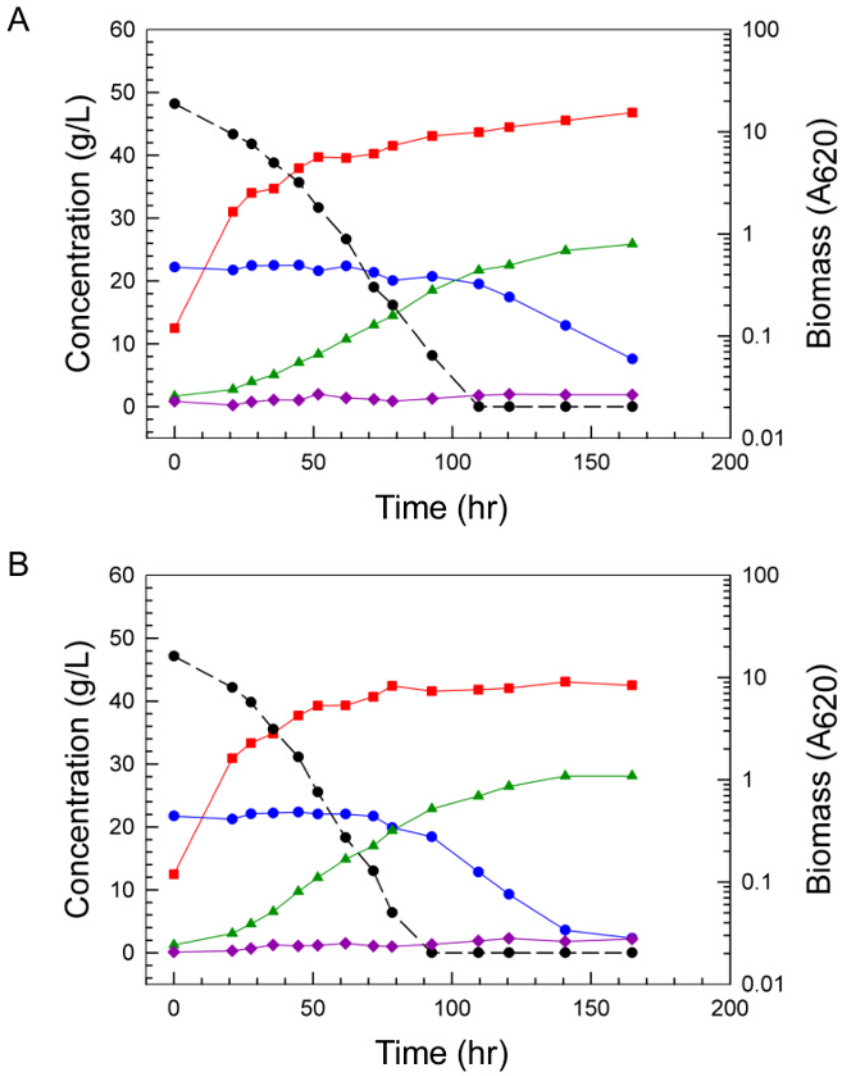

Figure 2: Improved batch fermentation of 6% glucan AFEX CSH. *Scheffersomyces stipitis* NRRL Y-7124 parent strain fermentation of 6% glucan AFEX CSH (**A**) is compared with adapted Colony 5 fermentation of 6% glucan AFEX-pretreated corn stover hydrolyzate (**B**). Symbols designate biomass (red square), glucose (black circle with dashed line), xylose (blue circle with solid line), ethanol (green triangle), and xylitol (purple diamond). (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

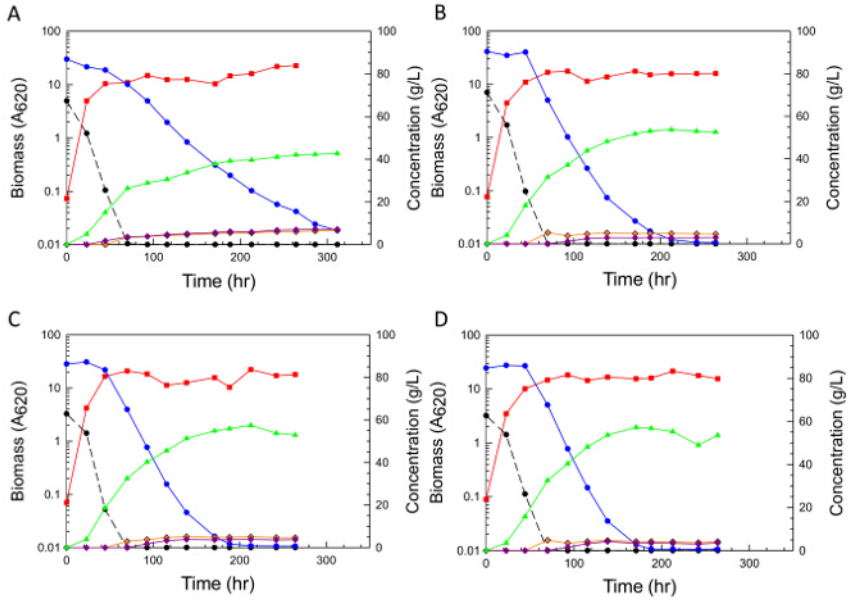

Figure 3: Reduced diauxic lag in defined medium with mixed sugars. Fermentation performances are compared in ODM with 66 g/L glucose and 87 g/L xylose for parent strain *S. stipitis* NRRL Y-7124 (**A**), the AFEX CSH adapted population derived from Y-7124 (**B**), single cell Colony 1 isolated from the adapted *S. stipitis* population (**C**), single cell Colony 5 isolated from the adapted population (**D**). Symbols designate biomass (red square), glucose (black circle with dashed line), xylose (blue circle with solid line), ethanol (green triangle), xylitol (purple diamond), and adonitol (gold diamond with black edge). (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

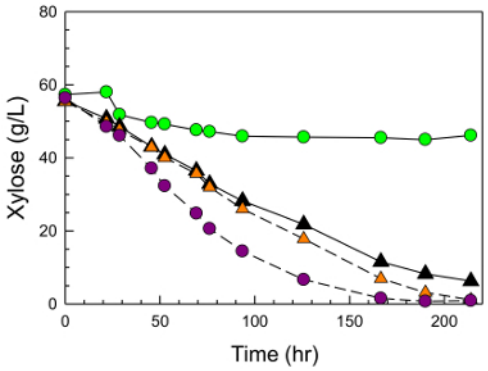

Figure 4: Ethanol resistant derivatives of Colony 5. Hydrolyzate tolerant Colony 5 was further developed by continuous culture selection on ODM containing xylose as sole carbon source and high levels of ethanol. Two derivative glycerol stock populations obtained early in the selection process (2A.1.53R, orange triangle and dashed line) and after UV irradiation of continuous culture inocula (2A.1.30R.2, purple circle and dashed line) are shown in comparison with the NRRL Y-7124 parent strain (green circle with solid line) and AFEX CSH tolerant Colony 5 (black triangle with solid line). Xylose uptake by dense populations of glucose-grown yeast (A_{620} = 50) in ODM with 40 g/L ethanol indicated that all adapted strains surpassed the unadapted parent in the ability to induce xylose metabolism. (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

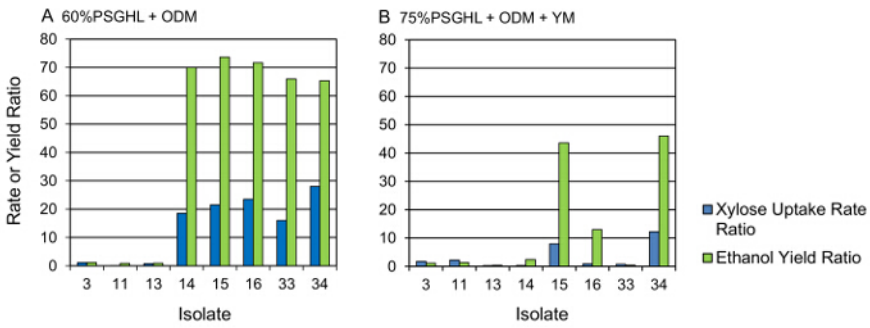

Figure 5: Ratio of performance improvement of tolerant isolate compared to parent. The performances of superior tolerant isolates are summarized relative to the control parent strain NRRL Y-7124 for each formulation of PSGHL (**A**, **B**). Performances were assessed in terms of xylose uptake rate (blue bars representing ratios of isolate to parent) and ethanol yield per sugar supplied (green bars representing ratios of isolate to parent). (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

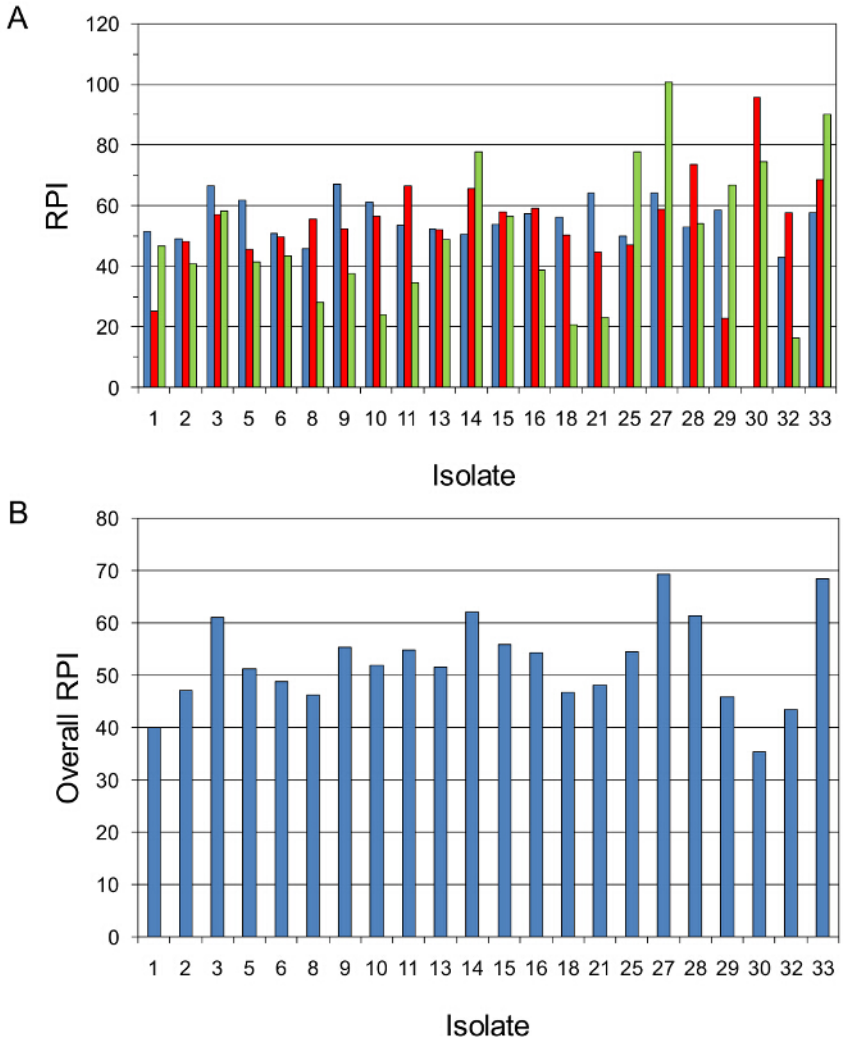

Figure 6: Isolate ranking based on RPI. The relative performance index (RPI) concept was applied to the performance results of the secondary screen in order to rank 33 isolates within each hydrolyzate type based on xylose uptake rate and ethanol yield per sugar supplied. (**A**) The relative ranking of any given isolate depended on the hydrolyzate type (P <0.001): SGH-N1(blue bars), SGH-N2 (red bars) and AFEX CSH (green bars). (**B**) The overall RPI calculated across all hydrolyzate types (light blue bars) indicated superior strains with most robust performance across different hydrolyzate conditions. (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

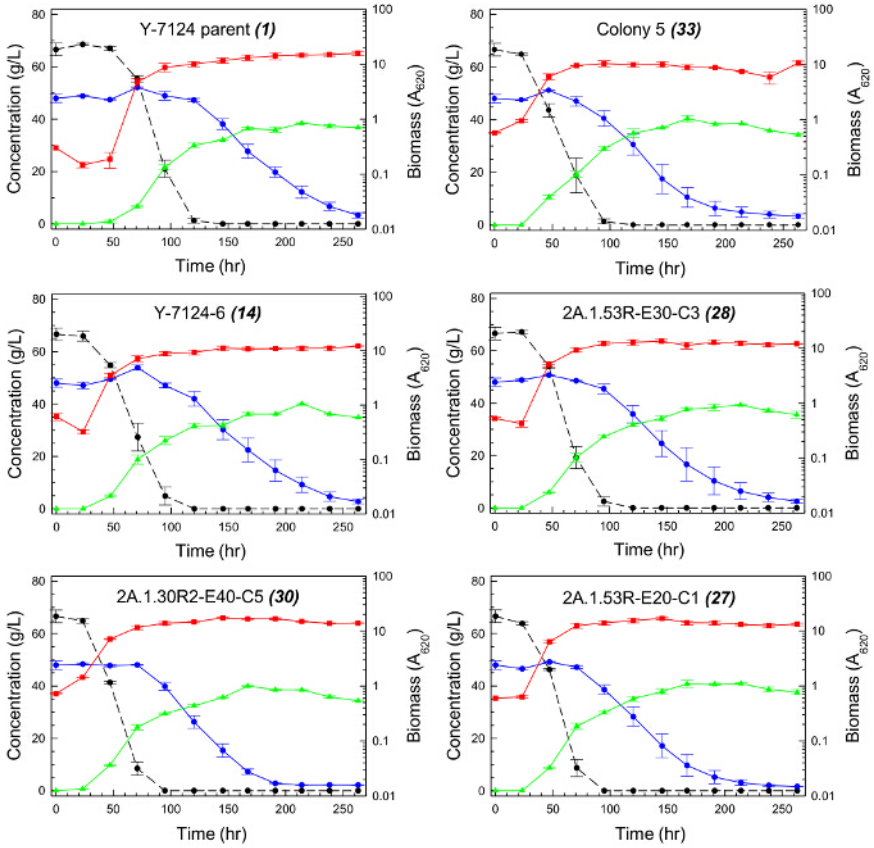

Figure 7: Comparative SGH fermentations of superior adapted isolates of *S. stipitis*. Superior adapted isolates and their parent strain NRRL Y-7124 are compared fermenting enzymatic hydrolyzates of dilute acid-pretreated switchgrass (20% solids loading) at 25 °C and initial pH 6.2 at low initial cell density. Time courses of biomass (red squares), glucose (black circles and dashed line), xylose (blue circles and solid line), and ethanol (green triangles) are shown. Error bars represent the range about the mean value marked by symbols. (Reproduced from Slininger *et al.*[18]) Please click here to view a larger version of this figure.

Table 1: Compositions of hydrolyzates used in cultivations. (Reproduced from Slininger *et al.*[18]) Please click here to download this table.

Table 2: Optimal defined medium for *Scheffersomyces stipitis* NRRL Y-7124[7] Please click here to download this table.

Table 3: Summary of superior tolerant *Scheffersomyces stipitis* strains for fermentation of hydrolyzates of plant biomass. (Reproduced from Slininger *et al.*[18]) Please click here to download this table.

Table 4: Comparative kinetics of isolates on switchgrass hydrolyzate SGH-N2 inoculated to initial A_{620} = 8.4 ± 2.5. Rates are normalized rates per unit absorbance during glucose or xylose consumption. (Reproduced from Slininger *et al.*[18]) Please click here to download this table.

Discussion

Several steps were critical to the success of the evolution process. First, it is key to choose appropriate selection pressures to drive the population evolution toward the desired phenotypes that are needed for successful application. The following selective stresses were chosen for *S. stipitis* development and applied at appropriate times to guide enrichment for the desired phenotypes: increasing strengths of 12% glucan AFEX CSH (which forces growth and fermentation of diverse sugars in the presence of acetic acid and low levels of furan aldehydes and other inhibitors); xylose fed continuous cultures with increasing ethanol concentration (which forces xylose enzyme induction to reduce diauxic lag); and increasing strengths of 20% solids loading PSGHL (which forces growth and fermentation of xylose in the presence of high acetic acid, furans, and other inhibitors). Second, it is important to preserve the evolving yeast populations by freezing glycerol stocks of population samples as the enrichment process progresses. Such snapshots of the population can be stored for periodic functional testing to document evolution progress and to allow subsequent isolations as desired, or to restart evolution processes after a hiatus. A third key step in the evolution procedure was to recover exceptional isolates by enriching selection culture populations or glycerol stocked populations on a convenient selective media (such as agar or micro-plates containing a stress gradient presenting a series of hydrolyzate and/or ethanol concentrations). Then surviving colonies growing under the most stressful condition can be picked to preserve for characterization later. These three basic steps can be repeated to pursue each additional desired phenotype selection pressure, or, alternatively, multiple pressures in a single cycle if appropriate. When the native parent yeast population is exposed to the various stresses, genetic diversity is expected to arise through natural or induced mutations, and continued exposure will allow natural selection to enrich for the individuals with the most beneficial mutations supporting competitive survival. It is expected that the selected phenotype will occur as a result of multiple genetic mutations. In the protocol above, mutations may be induced during UV treatment or potentially during exposure of yeast to hydrolyzates. Hydrolyzates are known to contain three

classes of compounds damaging to microorganisms: carboxylic acids, aldehydes and phenolics.[22] Reactive aldehydes, such as furfural and 5-hydroxymethylfurfural, can damage cells and cause an elevation of reactive oxygen species (ROS), generated typically in mitochondria. ROS are well known to cause DNA mutations in eukaryotic cells.[23,24] The phenolics present in hydrolyzates, though not previously known to be genotoxic, may promote and synergize the mutagenic effects of aldehydes and mutagenic ROS.[25]

The stepwise strategy of progressively challenging environments is expected to build evolved strains with a series of phenotype improvements, both targeted and also non-targeted. It is possible that certain non-targeted phenotypes may arise that are undesirable or unstable. In order to capture the most highly functioning and robust strains, an additional key step that must be taken is to evaluate and compare selected isolates for commercially relevant traits, reaching for both targeted and non-targeted phenotypes. To do this, isolate performances should be compared in a variety of application-oriented stress conditions, such as in hydrolyzates with different inhibitor challenges and nutrient formulations, allowing selection of best overall strains that are stable and robust to broad industrial lignocellulosic substrate variation. Additionally, a strain stability challenge can be incorporated into the screen as was done in the example by including preculturing steps involving initial yeast growth on two nonselective media, YM agar for several generations followed by the synthetic ODM with 50 g/L xylose for an additional 6-7 generations, giving opportunity for destabilization of desired culture traits prior to exposure to the challenge of performance in hydrolyzate. Based on significant performance features, such as ethanol yield and xylose uptake rate, isolates are then ranked in each different stress condition, such as each hydrolyzate environment tested, which may be different from the isolate's enrichment culture medium. The dimensionless RPI can be used to calculate the average overall relative performance and rank of isolates being compared. A dimensionless factor is appropriate to reflect relative performance in different types of hydrolyzates and based on different performance assessments, such as yields versus rates. This ranking procedure identifies strains that perform consistently well across broad variations in growth conditions and hydrolyzates and are apt to be both robust and genetically stable.

Various iterations and modifications of these basic steps may be necessary to accommodate the evolution of any microbial strain capable of specific or unique performance features on lignocellulosic hydrolyzates or other substrates of interest. In the *S. stipitis* example, it is important to note that supplementation of nutrients, including low-cost commercial sources, to hydrolyzates prepared at high solids loading was key to controlling the dynamic range of isolate performances for improved statistical separation during screening. The importance of nutrients to successful fermentation of concentrated inhibitory hydrolyzates has also been reported previously and is thought to be due to an elevated need for amino acids and other nutrients required for cell maintenance, redox balancing, and repair as a result of such stress conditions, especially during xylose utilization.[9,26,27] Additionally, if nutrients are too sparse or too profuse, differences in isolate performances may be hard to detect statistically due to all isolates doing exceedingly poorly, or all doing exceedingly well, respectively. Isolates capable of performing well under diverse conditions are expected to be reliable, or robust to variations in industrial conditions.

The main limitation of this protocol is that adaptive evolution and screening are very literal in outcome, in that "one will get what one enriches and screens for". Thus, the enrichment and screen conditions need to be designed to amplify and identify individuals, respectively, with the desirable changes in phenotypes while still retaining desirable pre-existing traits. Evolution may affect non-selected for traits that are critical for industrial performance (*e.g.,* growth factor requirements). For this reason, population enrichment progress for one trait needs to be periodically monitored for other traits as a method of troubleshooting for undesirable changes. For example, one of the unique traits of *S. stipitis* is its native ability to grow on and ferment xylose. All enrichment and screening substrates included xylose as sole or key contributing carbon source in the presence of inhibitors. Consequently, a common feature of all of the enrichment cultures in this example was that they directly selected for more rapid xylose utilizing strains, whose populations became more and more enriched in serial or continuous culturing because they were better competitors. As an intended result, improved strains were all capable of more rapid xylose uptake, but improvements to ethanol yield were much less dramatic than improvements in xylose uptake rate. The latter trend likely came about since ethanol yield by the parent strain was relatively high at around 0.3 g/g, or 60% of theoretical (0.51 g/g), as cell growth became stationary in hydrolyzates, leaving less room for improvement. In cultures, higher ethanol yields could be possibly selected against because ethanol is a growth inhibitor, which necessitated performance monitoring of the evolved population for this trait. Ethanol concentrations kept below the level for growth inhibition would tend to neutralize this as a selection factor. For *S. stipitis* NRRL Y-7124, 40 g/L ethanol halves specific growth rate while 64 g/L prevents growth entirely.[28] In ethanol challenged continuous cultures fed xylose, aeration was kept low and dilution rates were also kept low enough with ample xylose present to foster fermentation rather than respiration of ethanol and prevent enrichment for an unwanted ethanol use property. Additionally, nutrient supplements to the hydrolyzates used in performance screens were thoughtfully designed so as to avoid selecting for strains needing a costly rich nutrient supply, such as yeast extract. Supplements always included lowest cost commercial sources available, such as soy flour and urea, although richer components such as in YM were applied in tandem cultures for comparative testing, such as in the formulations of 60% and 75% PSGHL which are normally deplete of nitrogen. The ODM synthetic medium, used in preculture and performance culture screens, was originally designed for optimal performance of the parent strain *S. stipitis* NRRL Y-7124[7] in accordance with the culture medium optimization routine described by Traders Protein[20] which recommends defined ingredients, including vitamins, minerals, purines and pyrimidines, and amino acid sources to supply nutrients compatible with cost-effective industrial scale-up using commercial sources. A final recommendation in designing relevant strains, is to keep the design of the enrichment and screen conditions as relevant as possible to the expected industrial process conditions.

Cost-competitive renewable ethanol has long been pursued to reduce dependence on fossil fuels. To improve the ability of *S. stipitis* NRRL Y-7124 to tolerate, grow and ferment inhibitory hydrolyzates of lignocellulose, several researchers have used repetitive culturing in the xylose-rich liquor resulting from dilute acid-pretreatment of lignocellulosic biomass.[13,14,16,17] Over-liming to detoxify inhibitors was still needed to allow a reasonable level of performance of early adapted strains on hardwood and wheat straw pretreatment liquors.[13,14] Multiple repeated rounds of UV mutagenesis and genome shuffling have been applied to develop derivatives of *S. stipitis* NRRL Y-7124 with improved inhibitor tolerance and growth in hardwood spent sulfite liquor (HWSSL).[16,17] However, ethanol concentrations produced by these strains in HWSSL were under 10 g/L after 6 to 7 days. Using the techniques shown and described here, new evolved strains of *S. stipitis* NRRL Y-7124 were developed to meet phenotype goals needed for economical commercial use: fermentation of hydrolyzates at pH 5-6 without prior detoxification measures, such as over-liming, which leads to costly waste disposal; greatly reduced growth and diauxic lags; ethanol accumulations high enough for commercial distillation (>40 g/L ethanol); more rapid growth and ethanol productivity than previously reported for native yeast strains fermenting undetoxified hydrolyzates; and performance in diverse hydrolyzates at high solids loading, including appropriately soy nitrogen-supplemented SGH (which is otherwise nitrogen poor) and unsupplemented AFEX CSH. The improved strains developed using the novel aggressive approaches to evolution as embodied in the key steps summarized above, are expected to benefit the economics of producing ethanol from agricultural biomass by

lowering operating costs, capital costs, and energy and water inputs. This is the first strain evolution plan reported to yield adapted strains of *S. stipitis* demonstrating economically recoverable ethanol production on undetoxified hydrolyzates.

The novel strains of *S. stipitis* arising from each phase of the evolution process (**Figure 1**) are candidates for future studies to determine the genetic changes associated with specific selection pressures applied and the mechanisms underlying key attributes of improved hydrolyzate fermentation, such as inhibitor tolerance and reduced diauxic lag. Such valuable new knowledge will aid the engineering of next generation yeast biocatalysts and processes for conversion of lignocellulose to biofuels and other products. As new strains are derived, it will likely be beneficial to again pass the derivative population through a strain evolution protocol similar to that described here in order to select the most useful transformants for commercial use in hydrolyzates. Similarly, as new microbial strains are discovered with the potential to make a myriad of useful new products from renewable biomass, the evolution process could be further applied to improve strain robustness and productivity in hydrolyzates, potentially allowing novel bio-catalytic processes and products to be made available.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We would like to express our sincere appreciation to Drs. Kenneth Vogel, Robert Mitchell and Gautam Sarath, Grain, Forage, and Bioenergy Research Unit, Agricultural Research Service, Lincoln, NE for their kind supply of switchgrass for this project. We also thank U.S. Department of Energy for funding to VB through the DOE Great Lakes Bioenergy Research Center (GLBRC) Grant DE-FC02-07ER64494.

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