Primary souring: a novel bacteria-free method for sour beer production

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Abstract

In the beverage fermentation industry, especially at the craft or micro level, there is a movement to incorporate as many local ingredients as possible to both capture terroir and stimulate local economies. In the case of craft beer, this has traditionally only encompassed locally sourced barley, hops, and other agricultural adjuncts. The identification and use of novel yeasts in brewing lags behind. We sought to bridge this gap by bio-prospecting for wild yeasts, with a focus on the American Midwest. We isolated 284 different strains from 54 species of yeast and have begun to determine their fermentation characteristics. During this work, we found several isolates of five species that produce lactic acid and ethanol during wort fermentation: *Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans*, *Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*. Tested representatives of these species yielded excellent attenuation, lactic acid production, and sensory characteristics, positioning them as viable alternatives to lactic acid bacteria (LAB) for the production of sour beers. Indeed, we suggest a new LAB-free paradigm for sour beer production that we term “primary souring” because the lactic acid production and resultant pH decrease occurs during primary fermentation, as opposed to kettle souring or souring via mixed culture fermentation.

Keywords: *Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces japonicus*, *Wickerhamomyces anomalus*, lactic acid, sour beer, heterolactic fermentation

Chemical compounds studied in this article:

Lactic acid (PubChem CID: 612); Ethanol (PubChem CID: 702)

57 **Abbreviations:** ABV, alcohol by volume; DIC, differential interference contrast; EtOH, ethanol;
58 FG, final gravity; gDNA, genomic DNA; IBU, international bittering unit; LAB, lactic acid
59 bacteria; LASSO, lactic acid specific soft-agar overlay; N-J, neighbor-joining; OG, original
60 gravity; WLN, Wallerstein Laboratories nutrient; YPD, yeast extract, peptone, and dextrose
61

1. Introduction

Currently, we are in the midst of a global craft beer boom, with the number of small independent breweries growing at a tremendous pace (1). This has led to increased competition, not only with the large macrobrewers but among the craft brewers themselves. As such, there is a need in the industry to differentiate oneself from, minimally, other local breweries. This has fueled experimentation with the core beer ingredients of water (2), malted grain (3), hops (4) and yeast (5), as well as with various adjuncts. Much of this experimentation is also focused on locally sourced ingredients to capture terroir and bolster the local economy (6,7).

Despite this widespread experimentation, the isolation and use of novel yeasts for brewing has lagged behind that of the other ingredients. This is in part due to the easy availability of numerous ale and lager strains from reputable commercial suppliers such as White Labs, Wyeast, and Lallemand (8). However, focusing on two species, *Saccharomyces cerevisiae* for ales and *Saccharomyces pastorianus* for lagers, naturally limits the genotypic and phenotypic variation available in brewing strains. This also translates into a limited palette of aromatic and flavor compounds made by these strains, especially considering their extremely high evolutionary relatedness (9,10).

To overcome this constraint, several laboratories and breweries have begun to culture wild yeasts and characterize their beer fermentation capabilities. Most efforts have focused on wild ale and lager strains (11,12) to increase the available genetic diversity of strains that naturally display high ethanol tolerance. However, multiple strains of yeasts in the *Brettanomyces*, *Hanseniaspora*, *Lachancea*, and *Pichia* genera (13-15) have also been investigated as alternative species for the production of beer.

We also recently began bio-prospecting for wild yeasts with desirable brewing characteristics (5). Here, we report the collection of nearly 300 strains from 26 genera. During trial wort fermentations, we found that strains from five species (*Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans*, *Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*) were capable of heterolactic fermentation of sugar into lactic acid, ethanol, and CO_2. Larger-scale brewing with four strains demonstrated that these yeasts are highly attenuative, flocculate well, yield appreciable levels of lactic acid, and produce pleasant aromatic and flavor compounds. We suggest a new paradigm for sour beer production called "primary souring" that avoids the use of lactic acid bacteria (LAB) and instead relies solely on lactic acid production by a heterofermentative yeast during primary fermentation.

2. Materials and methods

2.1. Strains, media, and other reagents

S. cerevisiae strain WLP001 was purchased from White Labs (San Diego, CA). Wild strains were isolated as described in (5). All yeast strains were routinely grown on yeast extract, peptone, and dextrose (YPD; 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) plates containing 2% (w/v) agar at 30°C and in YPD liquid culture at 30°C with aeration unless otherwise noted. Wallerstein Laboratories nutrient (WLN) agar contained 4 g/L yeast extract, 5 g/L tryptone, 50 g/L glucose, 0.55 g/L KH_2PO_4, 0.425 g/L KCl, 0.125 g/L $CaCl_2$, 0.125 g/L $MgSO_4$, 2.5 mg/L $FeCl_3$, 2.5 mg/L $MnSO_4$, 22 mg/L bromocresol green, and 15 g/L agar. All strains were stored as 15% (v/v) glycerol stocks at -80°C. Media components were from Fisher

Scientific (Pittsburgh, PA, USA) and DOT Scientific (Burnton, MI, USA). All other reagents were of the highest grade commercially available.

2.2. Strain identification and phylogenetic analysis

To identify wild yeasts at the species level, frozen stocks were streaked onto YPD plates and incubated at 30°C until single colonies formed (18-48 h). Colonies were then picked into microcentrifuge tubes containing 100 µL of lysis solution (0.2 M LiOAc and 1% SDS) and incubated in a 65°C water bath for ≥15 min to lyse the cells. After 300 µL of 100% isopropanol was added to the tubes, they were mixed by vortexing, and the cell debris and genomic DNA (gDNA) were pelleted in a microcentrifuge for 5 min at maximum speed. The supernatant was decanted, and remaining traces were completely removed from the pellets by aspiration. The gDNA was resuspended in 50-100 µL TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA), and a 1-min spin at maximum speed was used to pellet the cell debris to clarify the DNA solution. The variable D1/D2 portion of the eukaryotic 26S rDNA was then amplified by PCR from the gDNA templates using oligos NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) (11) and the following cycling conditions: 98°C for 5 min; 35 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 10 min. The PCRs were assessed for D1/D2 amplification by running 10% of the reaction volume on 1% (w/v) agarose gels at 100 V (560 bp expected product size). The amplified DNA was then purified using a PCR Purification Kit (Thermo Scientific, Waltham, MA) and quantified using a BioTek Synergy H1 plate reader. The DNA was sequenced by ACGT, Inc. (Wheeling, IL) using primer NL1, and the sequence was used to query the National Center for Biotechnology Information nucleotide database with the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

After species identification, the phylogenetic relationships among the isolated strains of *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* were determined by aligning their 26S rDNA sequences using ClustalX (16). The alignments were iterated at each step but otherwise utilized default parameters. ClustalX was also used to draw and bootstrap neighbor-joining (N-J) phylogenetic trees using 1000 bootstrap trials; the trees were visualized with TreeView v. 1.6.6 software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The *Schizosaccharomyces pombe* rDNA sequence (GenBank accession HE964968) was included in the alignments as the outgroup, and this was used to root the N-J tree in TreeView. WLP001 was included to determine the relatedness of the wild strains to a commercially available ale yeast.

2.3. Test fermentations

For laboratory-scale fermentations, select yeast strains were streaked for single colonies onto YPD plates as described above and grown to saturation in 4 mL of YPD liquid medium overnight at 30°C with aeration. The cell count of the starter cultures was approximated by measuring the OD_{660} and converting that value to cells/mL as described at http://www.pangloss.com/seidel/Protocols/ODvsCells.html. In most cases, the saturated overnight cultures reached densities of ~$5x10^8$ cells/mL. These starter cultures were then used to inoculate ~400 mL of blonde ale wort in 500 mL glass bottles capped with drilled rubber stoppers fitted with standard plastic airlocks. The wort was produced by mashing 65.9% Pilsner (2 Row) Bel and 26.9% white wheat malt at 65°C (149°F) for 75 min in the presence of 1 g/bbl $CaCO_3$ and 1.67 g/bbl $CaSO_4$ to yield an original gravity (OG) of 1.044. During the boil, 7.2% glucose was added, as well as Saaz hops to 25 international bittering units (IBUs). The fermentation cultures were incubated at 22.3±0.3°C (~72°F) for 2 weeks. Prior to bottling into

151 standard 12-oz brown glass bottles, their final gravity (FG) was measured using a MISCO digital
152 refractometer (Solon, OH), and pH was measured using an Accumet AB150 pH meter (Fisher
153 Scientific). Bottle conditioning was conducted as in (17) at room temperature for ≥2 weeks.

154 Small-batch fermentations were performed at Mainiacal Brewing in Bangor, ME. To
155 produce the test wort, 93.4% two-row base malt and 6.6% carapils were mashed at 66.7°C
156 (152°F) to yield an OG of 1.046. During the boil, Loral hops were added to a final concentration
157 of 5.3 IBUs. The wort was then chilled and split into 5-gal portions in separate carboys.
158 Approximately $1x10^{11}$ cells of the indicated yeast strains were used to inoculate the carboys and
159 allowed to ferment under anaerobic conditions at 21.7°C (71°F) for 1 month. Gravity
160 measurements were taken both with a hydrometer and refractometer by standard methods. The
161 final pH was recorded prior to bottling and bottle conditioning as above.

162 *2.4. Yeast morphology analysis*

163 To determine colony morphology, the yeast strains were first grown with aeration
164 overnight at 30°C in liquid YPD medium. Small volumes were then spotted onto YPD and WLN
165 plates and streaked for single colonies. The plates were incubated at 30°C for ~48 h before
166 imaging with an Epson V37 flatbed scanner. The same liquid cultures were sampled for
167 microscopic analysis in the Indiana University Light Microscopy Imaging Center. Briefly, ~5 μL
168 samples of cell culture were spotted onto 25 x 75 mm (1.0 mm thick) glass slides and covered
169 with a 22 x 22 mm no. 1.5 cover slip. The live cells were then observed by differential
170 interference contrast under oil immersion at 1000x magnification using a Nikon NiE microscope.
171 Digital images of the fields of view were collected using Nikon Elements software.

172 *2.5. Lactic acid specific soft-agar overlay (LASSO)*

The production of lactic acid by yeast cells was assayed as described in (18). Briefly, cells were grown overnight in liquid YPD medium at 30°C with aeration. Then, 2 µL of each culture was spotted onto YPD10 plates (YPD agar supplemented with glucose to a final concentration of 10% w/v), allowed to absorb, and incubated overnight at 30°C. The plates were then covered with 6.5 mL soft-agar (0.5% agar in 300 mM Tris and 187 mM glutamate, pH 8.3). Upon solidification of the soft agar, a second soft-agar overlay was prepared by mixing 3.2 mL 1% agar with 3.2 mL of a staining solution composed of 30 mM Tris/18.75 mM glutamate (pH 8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL phenazine methosulfate, 7 U glutamate pyruvate transaminase, and 7 U L[+]-lactate dehydrogenase (all LASSO components were from Sigma-Aldrich, St. Louis, MO). Strains producing lactic acid formed purple halos within 10 min, the color of which darkened with increasing incubation time at room temperature. Cells not producing lactic acid never formed halos.

2.5. Multi-well lactic acid production assay

Because only a small number of strains can be tested for lactic acid production on a single plate in the LASSO assay, we also adapted it for use in multi-well plates. Briefly, individual strains were grown overnight in 100 µL YPD10 medium in the wells of 96-well plates at 30°C with aeration in a BioTek Synergy H1 plate reader. To avoid evaporation of the medium, 50 µL mineral oil was used to overlay each well. Then, 100 µL of staining solution (30 mM Tris/18.75 mM glutamate (pH 8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL phenazine methosulfate, 1 U/mL glutamate pyruvate transaminase, and 1 U/mL L[+]-lactate dehydrogenase) was added to each well and mixed by agitation in the plate reader. The reaction

194 proceeded for ≥ 10 min at room temperature, and the presence of lactic acid was indicated by the
195 gold colored solution turning green (and eventually blue with extended incubation).

196 *2.6. Gas chromatography-mass spectrometry (GC-MS) analysis of lactic acid*

197

198

199 **3. Results**

200 *3.1. Establishment of the yeast bank*

201 We previously reported an initial description of our bio-prospecting and characterization
202 of wild yeasts for use in the brewing industry (5). Here, we present the updated culture
203 collection. In all, 284 strains (Supplementary Table 1) from 54 different species in 26 genera
204 (Table 1) were collected, mostly from the American Midwest. Over 68% (195 strains) were from
205 various locations in Indiana alone (Supplementary Table 1). Species known to have brewing
206 potential, *e.g.*, *S. cerevisiae* and *B. bruxellensis*, were isolated from many sources, and the 37 *S.*
207 *cerevisiae* strains recovered made it the most frequently isolated species (Table 1). However,
208 these yeasts represented a minority (~13% *S. cerevisiae* and < 3% *B. bruxellensis*) of the total.
209 Instead, species in the *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Pichia*, *Torulaspora*, and
210 *Wickerhamomyces* genera dominated (Table 1).

211 To determine the relative usefulness of these strains in beer brewing, small laboratory-
212 scale beer fermentations were performed for each isolate. Most strains were able to metabolize
213 maltose (and other wort sugars) into ethanol and carbon dioxide, but the strains greatly varied in
214 their levels of attenuation, ability to flocculate, and in the sensory profiles of the aromatic and

flavor compounds that they produced (data not shown). Interestingly, these variations were noted both between species and among isolates of the same species, even when two distinct strains of the same species were isolated from one sample. Notable exceptions to this were all of the *Pichia* species. The sensory profiles of beers fermented with these yeasts were uniformly abhorrent, with the most colloquial but apt descriptor being "demon sulfur fecal juice." Regardless, many of the other yeasts displayed brewing potential that was commensurate with our control *S. cerevisiae* ale yeast strain WLP001. Reports of the detailed analysis of the brewing characteristics of exemplary single strains and strain groups are forthcoming. Herein though, we focus on a group of yeasts that appeared to be capable of heterolactic fermentation of sugar into lactic acid, ethanol, and CO_2.

3.2. Five heterofermentative yeast species.

During our sensory analyses of the laboratory-scale fermentations above, we noted that many of the strains were producing beers that were characterized as tart or sour (Table 2), akin to styles that are produced with the aid of LAB (19). When we grouped these strains, we found that they were all members of five species: *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* (Supplementary Table 1 and Fig. 1). To determine if this apparent heterofermentative activity was specific to evolutionarily closely related yeasts, we aligned the sequences of the D1/D2 variable region of their ribosomal DNA and plotted a phylogenetic tree. As shown in Figure 1, three of the species (*H. vineae*, *L. fermentati*, and *L. thermotolerans*) are closely related to ale yeast (WLP001), but the other two (*S. japonicus*, and *W. anomalus*) form more distinct clades.

Regardless of their evolutionary relationships, the strains listed in Table 2 and other isolates of the same species (data not shown) varied in their fermentative activities. Their levels of attenuation varied from 40-83%, with decreases in the initial pH of 5.0 to as low as 3.21. Although some of these differences may be attributable to differences among the species themselves, intra-species differences were also noted, especially during sensory analyses. For instance, *L. thermotolerans* YH73 produced a "very sour" flavor with berry notes, but the same beer fermented with *L. thermotolerans* YH79 was characterized as only "slightly tart" yet clean and rounded (Table 2).

3.3. Characterization of strains WYP39, YH72, YH82, YH140, and YH156

Because the trial fermentations above were performed in a high-throughput manner, we chose representative strains for more in-depth investigation. We compared their colony and cellular morphologies, sought to verify that they were producing lactic acid, and performed larger-scale fermentations. Figure 2A shows representative colony morphologies for the strains grown on YPD and WLN agar. The wild *S. cerevisiae* strain YH166 was included for comparison. On YPD plates, all strains produced colonies that were creamy white, round, and raised. The *L. fermentati* WYP39 and *L. thermotolerans* YH140 colonies were also glossy, while the others had comparatively matte surfaces. Based on colony size, the *H. vineae* YH72, *W. anomalus* YH82, and (especially) *S. japonicas* YH156 strains grew slower on YPD medium than the other species. However, all strains grew at similar rates on WLN agar. All of the species also took up the bromocresol green pH indicator dye from the WLN medium, with YH166 and YH140 attaining a pale mint green colony color, YH156 assuming a green shade, and WYP39, YH72, and YH82 colonies turning dark green. The YH72 and YH82 colonies additionally displayed pale margins on WLN plates.

We next observed the cellular morphologies of the six species above using differential interference contrast (DIC) microscopy (Fig. 2B-G). As expected, all strains except YH156 appeared as typical round-to-ellipsoid budding yeast, though YH72 failed to display the "bowling pin" shape usually associated with apiculate yeast like *H. vineae*. Additionally, the YH72 and YH82 cells displayed the propensity to clump together (Fig. 2C and D), even after dilution and vigorous vortexing. This may be an effect of growth with aeration, as these strains were no more flocculant during fermentation than WYP39 (Table 3) or YH166 (data not shown). The YH156 cells were much larger than the other species analyzed and displayed the usual *Schizosaccharomyces* rod-shaped morphology (Fig. 2F). We also observed evidence of microhyphae (20) among these cells. This mycelial mode of growth may explain the excellent flocculation of YH156 during fermentation (Table 3) (21).

3.4. Strains WYP39, YH72, YH82, YH140, and YH156 produce lactic acid

We further sought to determine if strains WYP39, YH72, YH82, YH140, and YH156 were truly producing lactic acid during fermentation, rather than one or more other secondary metabolites that yield a tart/sour flavor (22). Using the LASSO assay for lactic acid production by yeast (18), we found that all five strains produced lactic acid (denoted by dark halos in Fig. 3A), similar to the *Lactobacillus plantarum* positive control. In contrast, the *S. cerevisiae* WLP001 negative control failed to generate a halo. Because the LASSO assay can only be used for a limited number of strains on a single plate, we adapted it into a multi-well plate assay (Fig. 3B). Here, lactic acid production is evident by the golden-colored assay medium turning green, as indicated by the LAB controls. Again, WLP001 failed to generate detectable lactic acid, as did a common research strain of *E. coli*. However, multiple tested isolates of *L. thermotolerans*, *L. fermentati*, *H. vineae*, *S. japonicus*, and *W. anomalus* did test positive for lactic acid production.

Some individual *L. thermotolerans* strains failed to generate lactic acid (wells 2 and 4) or did so slowly, as certain wells were just beginning to turn green (well 3) when the image in Figure 3B was acquired. These results correspond with sensory analysis of beers fermented with the various *L. thermotolerans* strains, which ranged from not sour to very tart (Table 2 and data not shown).

3.4. Analysis of beers fermented with lactic acid-producing yeasts

To monitor the activities of WYP39, YH72, YH82, and YH156 in larger-scale fermentations, we inoculated these strains into glass carboys containing 19 L (5 gal) each of an identical blonde wort. Because the beer brewing capabilities of a *L. thermotolerans* strain were recently described (14), we omitted strain YH140 from these assays. All of the strains had short lag times (*i.e.*, the approximate time from inoculation to the first visible signs of fermentation) ranging from 6-14 h (Table 3). These lag times to fermentation were similar to that of WLP001 inoculated in a similar blonde wort (~12 h, data not shown). However, the kinetics of the full fermentation differed for each lactic acid-producing yeast. WYP39 had the shortest lag time and fermented rapidly for 2 weeks, at which point it slowed considerably and required an additional 2 weeks to reach a final gravity of 0.099 (Table 3). YH72 was a slow and steady fermenter, attaining a terminal gravity of 1.000 after 3 weeks. YH82 was similar, but required a full 4 weeks to reach a final gravity of 1.001.

The YH156 strain displayed the most variant fermentative characteristics. After reaching a vigorous state of fermentation at 14 h (Table 3), popcorn-like clusters of cells formed and floated around within the fermenter (Supplemental Fig. 1). Eventually, they settled into a mountainous pile against one side of the carboy before compacting down into a yeast slurry with a typical appearance on the bottom of the fermenter. The fermentation reached a final gravity of

304 0.099 approximately 27 days after inoculation. The final pH of each beer was recorded and
305 varied from a low of 3.20 to a high of 3.74. Sensory analyses of each beer were conducted
306 (Supplemental Fig. 2), and the tasting notes are discussed in Sections 4.1-4.5 below.

307 We also quantified the concentration of L-lactic acid present in each beer by GC-MS
308 (Fig. 3C). We used Cauldron, a commercially available sour beer made by mixed fermentation of
309 yeast and LAB (17), as a positive control for lactic acid production; it contained 100.54 mM
310 lactate. Based on a lactate standard curve, WYP39, YH72, YH82, and YH156 produced 10.02,
311 35.69, 29.05, and 50.09 mM lactate, respectively. These results contrast with those from the
312 LASSO assay in Figure 3A, where YH156 displayed the least evidence of lactic acid production.
313 Because the lactic acid production occurred in the presence of oxygen in the LASSO assay, this
314 may indicate that strain YH156 is Crabtree negative or only weakly Crabtree positive, *i.e.*,
315 requiring anaerobic conditions for fermentation (see (23) and references therein). It should also
316 be noted that the beers analyzed by GC-MS were fermented in a brewery that utilizes LAB, and
317 thus, it remains a formal possibility that they may have been inadvertently contaminated by other
318 organisms that can generate lactic acid. However, the results in Figures 3A and 3B are from pure
319 cultures of WYP39, YH72, YH82, and YH156, which still yield lactic acid during fermentation
320 in the presence of antibiotics or 75 IBU wort in a laboratory setting (data not shown), strongly
321 suggesting that LAB contamination is not the source of the lactic acid.

322 **4. Discussion**

323 The next phase in the "local" movement in the beer industry will be the isolation and use
324 of local yeast strains in brewing. Indeed, in the U.S., nearly all commercially available ale and
325 lager strains are of European origin, so no American beer will ever truly be local without the

inclusion of New World yeast. Here, we detailed our regional yeast bio-prospecting efforts and the initial characterization of nearly 300 strains for use in fermentation. Within this strain bank, we uncovered five species that generate lactic acid and ethanol during primary fermentation and suggest that they can be used in a novel, LAB-free beer souring method (see Section 4.6. below).

4.1. H. vineae

As the species name suggests, *H. vineae* is typically associated with wine, where it has previously been investigated alone and in combination with *S. cerevisiae* for grape must fermentation (24-26). The strains previously tested are notable for the production of high levels of 2-phenylethyl acetate, which is an aromatic compound that lends floral, fruity, and/or honey-like notes to wine (25). Although yeasts in the *Hansenia* genus are the predominant species found on grapes (27-29), they are also found elsewhere. For instance, *H. vineae* has been isolated from agave plants, must, and cooked agave, as well as throughout tequila distilleries (reviewed in (30)). Here, we enriched for *H. vineae* strains from the bark of oak, sycamore, and ash trees, blueberries, and grapes (Supplementary Table 1).

To our knowledge, this is the first report of pure cultures of *H. vineae* being used to ferment beer. Strain YH72 ferments slowly relative to typical commercially available ale yeasts, but reached high levels of attenuation after only two weeks (Table 2) and further attenuated with additional fermentation time (Table 3). The beers produced by short fermentations with YH72 were slightly sour but clean and highly drinkable, with notes of apple cider. Longer fermentation yielded very sour beer with a pH (3.23) and acidic bite reminiscent of beers produced with LAB, as well as stone fruit notes (Table 3, Supplementary Fig. 2). Preliminary experiments also indicate that repeated exposure to wort fermentation conditions may adapt YH72 to attenuate

348 more quickly (M. Bochman, observations). Based on these results, other closely related species,
349 such as *H. guilliermondii*, *H. occidentalis*, *H. opuntiae*, *H. osmophila*, *H. uvarum*, and *H.*
350 *valbyensis*, should be investigated for their use as brewing strains and the production of lactic
351 acid. We isolated several strains from three of these species (Table 1) and plan to characterize
352 their fermentative capabilities.

353 *4.2.* L. fermentati

354 Very little is known about *L. fermentati*, especially with regard to beverage fermentation.
355 In the environment, *L. fermentati* is generally associated with leaves and decaying plant matter
356 (31,32). We isolated our strains from the bark of live trees such as oak, hickory, and birch
357 (Supplementary Table 1). Industrially, this species of yeast has also been found in fermented
358 (wine (33), cachaça (34), and water kefir (35)) and non-fermented beverages (coconut water and
359 fruit juices (36)). However, its effects on the sensory characteristics of these beverages are
360 largely unknown.

361 As with *H. vineae*, this also appears to be the first report of beer fermented with pure
362 cultures of *L. fermentati*. Our laboratory-scale test fermentations indicated that strain WYP39
363 displayed decent wort attenuation for a wild strain (60%, Table 2), and longer fermentation in a
364 larger-scale fermentation yielded a dry product (Table 3). The final pH was modest compared to
365 other sour beers (Table 3), creating a flavor that was more tart than sour, but this was
366 accentuated by light pineapple and mango flavors. There are many species in the *Lachancea*
367 genus (37). Based on the desirable brewing characteristics of *L. fermentati* and *L. thermotolerans*
368 (discussed below in Section 4.3. and in Domizio *et al*. (14)), it will be interesting to assess the

369 activities of these other species during beer fermentation. Thus, our four isolates of *L. kluyveri*
370 (Table 1) will be the focus of future work.

371 *4.3.* L. thermotolerans

372 Like the closely related *L. fermentati*, *L. theromtolerans* also colonizes leaf surfaces (32),
373 as well as insects (38,39) and sugarcane (40). We isolated our strains from the barks of numerous
374 tree species, mulberries, bell pepper, and persimmon fruit (Supplementary Table 1). Various
375 strains of *L. theromtolerans* have been studied for their effects on wine fermentation (reviewed
376 in (28)), though usually in co-fermentations with *S. cerevisiae* (*e.g.*, (41,42). Recently, *L.*
377 *thermotolerans* strain Lt101 was shown to be a viable yeast for beer production (14), and the
378 same group also found that three *L. thermotolerans* strains including Lt101 produce lactic acid
379 during fermentation. Although this is similar to our observations (Table 2 and data not shown),
380 the strains investigated by Domizio *et al*. (14) only decreased the pH of wort from 5.66 to 4.28-
381 3.77 during fermentation. Most of their experiments yielded final pH values in the 4.17-4.3
382 range, however, which is similar to the pH decrease caused by *S. cerevisiae* UCD 915. Our *L.*
383 *thermotolerans* isolates that produced noticeably tart beers reached terminal pH values of ~3.35
384 (Table 2 and data not shown). These discrepancies are likely due to differences in the
385 experimental set ups, as well as strain-to-strain variability, which is discussed in Section 5
386 below.

387 *4.4.* S. japonicus

388 *S. japonicus* is closely related to *S. pombe*, which was originally isolated from African
389 millet beer (reviewed in (43)). *S. japonicus* itself was first isolated from strawberries in Japan
390 (44), and we recovered two strains from the bark of an oak tree in northeastern Pennsylvania

(Supplementary Table 1). Unlike the other yeast species investigated here, *S. japonicus* is a fission yeast rather than a budding yeast (Fig. 2E). This difference in physiology is borne out of genomic differences, as *S. japonicus* is as evolutionarily distant from *S. cerevisiae* as each yeast is from humans (45).

Regardless, *S. japonicus* is associated with indigenous fermented beverages (*e.g.*, kaffir beer, plantain beer, palm wine, sugar cane wine, and sake) around the world (46), wine production (47), and was isolated from spontaneously fermented beer in North Carolina (48). However, no characterization of the beer is available. Thus, this is the first rigorous report of *S. japonicas* used for primary fermentation of beer. The attenuation levels of strain YH156 were excellent in both laboratory- and large-scale fermentations (Table 2 and 3), and the aroma and flavor profiles of these beers included common descriptors of sour, fruity, and stone fruit. Individual tasters identified green apple Jolly Rancher, tart apple, pear, pineapple, and peach notes.

The *Schizosaccharomyces* genus includes four member species: *S. cryophilus*, *S. japonicus*, *S. octosporus*, and *S. pombe*. We isolated two *S. japonicus* and four *S. pombe* strains (Table 1), but only the *S. japonicus* YH156 strain yielded appreciable lactic acid production during our trial fermentations (Tables 2, 3, and data not shown). Regardless, we believe that it is worthwhile to test other strains of these species, as well as *S. cryophilus* and *S. octosporus*, for their use as in the primary fermentation of beer.

4.5. W. anomalus

W. anomalus, previously known as *Saccharomyces anomalus* (49), has multiple roles in the biotechnology, agriculture, and food production fields. It is often found in association with

413 grain and is especially useful to inhibit storage molds during malting (50). Concerning beverage
414 fermentation, *W. anomalus* is generally referred to as a beer spoilage organism (51), but it is also
415 necessary for cocoa and coffee bean fermentation (52). We isolated 17 strains of *W. anomalus*
416 from diverse sources: fruit, tree bark, beer, flowers, soil, and spent grain (Table 1 and
417 Supplementary Table 1).

418 Although *W. anomalus* has been investigated for its use in apple wine and hard cider
419 production (53,54), the only work involving beer has focused on the spoilage properties of this
420 species (51). As with *S. japonicus* YH156 above, the YH82 strain of *W. anomalus* yielded
421 excellent attenuation at both fermentation scales tested (Tables 2 and 3). This strain produced a
422 less intense sour character than others, but the beer was characterized as clean, aromatic, and
423 fruity with notes of pear, apple, and apricot. Of all of the lactic acid-producing yeasts described
424 herein, *W. anomalus* is perhaps the most exciting because the *Wickerhamomyces* clade contains
425 over a dozen species, with more being discovered and characterized with regularity (55).
426 Although we did not isolate closely related species to *W. anomalus*, our small collection of 17
427 strains is nonetheless an excellent place to begin to determine the usefulness of these yeasts in
428 beer fermentation.

429 *4.6. Primary souring*

430 There are two general methods by which sour beers are produced: kettle souring and
431 mixed culture fermentation (Fig. 4) (19). Kettle souring is the more rapid and modern technique.
432 During kettle souring, unhopped or lightly hopped wort is produced in a brew kettle as normal,
433 but then it is only partially cooled to approximately 40-45°C. This temperature favors the growth
434 of LAB, which can be introduced by inoculation with pure cultures or the addition of grain. The

435 LAB then sour the wort in the brew kettle to the desired pH, which usually occurs over the
436 course of 24-48 h. The soured wort in the kettle is ultimately boiled a second time to kill the
437 LAB, and hops can be introduced at this point. The wort is then transferred to a fermenter and
438 (typically) inoculated with *S. cerevisiae* for primary fermentation. Although this method affords
439 brewers tight control over acid production (souring can be stopped at any time via boiling) and is
440 less time consuming that mixed culture fermentation (below), it also has inherent weaknesses.
441 First, the entire souring process occurs in the brew kettle, so it prevents additional wort
442 production in that vessel. Indeed, kettle souring is often relegated to weekends when small
443 breweries are otherwise not in production mode. Second, boiling the wort after it has been soured
444 drives off volatile aromatics that may also have been produced during souring, yielding beers
445 that are described and criticized as lacking in depth and character. To combat some of these
446 sensory downsides, some brewers are now barrel aging kettle sours to impart oak complexity to
447 the final beers.

448 Souring by mixed culture fermentation is the more traditional method. In this process
449 (Fig. 4), lightly hopped wort is produced in the brew kettle and transferred to a fermenter. There,
450 it can be inoculated with *S. cerevisiae* for primary fermentation. In some cases, LAB are added
451 prior to or concurrently with *S. cerevisiae* (or *Brettanomyces* spp. in 100% "Brett" beers). If the
452 LAB are added at this stage, souring begins during primary fermentation. After the yeast has
453 attenuated the beer to the desired level, it is then barrel aged for months or years until it attains a
454 low pH and complex flavor profile. Barrel aging is another stage at which LAB and
455 *Brettanomyces* spp. can be added (either resident in the barrels or as pure inocula) to induce
456 souring. Mixed culture souring produces more complex and nuanced flavors in beer than kettle

457 souring, but it suffers from a huge time lag from wort production to the final beer, and it requires
458 a large space dedicated to housing the barrels.

459 Here, we propose a third paradigm for sour beer production that we call primary souring.
460 In this method, the wort is inoculated with a yeast capable of heterolactic fermentation rather
461 than *S. cerevisiae*, and souring occurs during primary fermentation in the absence of LAB (Fig.
462 4). The yeast strains described in Tables 2 and 3 and in the text above did not display rapid
463 fermentation kinetics like commercially available ale yeasts, but they still completed
464 fermentation within a month, displaying excellent levels of attenuation and medium-to-high
465 flocculation (Table 3). Further, the sensory profiles of the beers were superior to kettle soured
466 beer, displaying both lactic tartness and fruity aromatic and flavor notes. Compared to the sour
467 production methods above, primary souring is beneficial in that it frees up the brew kettle and
468 does not require lengthy aging in barrels, though oak aging is a possibility after primary
469 fermentation (Fig. 4). Perhaps most alluringly, primary souring does not require the introduction
470 of bacteria into the brewery, and preliminary tests suggest that *H. vineae*, *L. fermentati*, *L.*
471 *thermotolerans*, *S. japonicus*, and *W. anomalus* can be eliminated as easily as *S. cerevisiae* from
472 brewing equipment using standard clean-in-place protocols (data not shown). As is typical of
473 yeasts, these species are also hop tolerant, enabling more liberal use of hops in wort production
474 for sour beers. However, it should be noted that yeast growth can be inhibited by hop iso-α-acids
475 in acidic milieus (56), so the absolute levels of hop tolerance will likely vary by strain and the
476 desired pH of the sour beer.

477

478 **5. Conclusions and Outlook**

We set out to isolate and characterize new yeasts for use in beer fermentation, to mine the natural treasure trove of wild strains for novel aromas and flavors that have been missed by focusing on the currently used highly genetically related brewing yeast strains of *S. cerevisiae*. Here, we reported the results of our local bio-prospecting efforts and highlighted the discovery of a set of yeast species that produce both lactic acid and ethanol during primary fermentation. It is unclear how widespread this heterolactic fermentation phenotype is among ethanol-tolerant yeasts, but its presence in the fission yeast *S. japonicus* and budding yeasts like *H. vineae*, which are separated by ~1 billion years of evolution (45), may indicate that heterolactic fermentation is an ancient and conserved metabolic process among single-celled fungi. Arguing against this hypothesis is the lack of detectable lactic acid production by related strains of the same species, *e.g.*, differences among *L. thermotolerans* isolates ((14) and data not shown). Perhaps the genes enabling lactic acid production are on an extrachromosomal element only found in certain strains, or they may be epigenetically silenced in some cases but not others. The latter possibility is intriguing given the prion-based heritable changes in sugar metabolism that are caused by bacteria communicating with yeast cells via lactic acid (57,58). Regardless, it is our hope that the strains described above and the primary souring process put forth here will add strength to the already growing sour beer movement in the U.S. and abroad.

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659

660 **Tables**

661 **Table 1.** Wild yeast species diversity and number of isolates.

Genus	Species	Isolates
Aureobasidium	*pullulans*	2
Brettanomyces	*bruxellensis*	7
Candida	*carpophilia*	1
	intermedia	1
	sake	2
	tropicalis	6
	zemplinina	1
	zeylandoides	1
Clavispora	*lusitaniae*	3
Cryptococcus	*albidus*	1
Cyberlindnera	*fabianii*	5
	rhodanensis	1
Debaryomyces	*hansenii*	7
Hanseniaspora	*opuntiae*	1
	uvarum	12
	valbyensis	1
	vineae	11
Issatchenkia	*orientalis*	1
	terricola	3
Kazachstania	*unispora*	1
Kluyveromyces	*lactis*	10
	marxianus	10
Kodamaea	*ohmeri*	2
Kwoniella	*mangroviensis*	1
Lachancea	*fermentati*	8
	kluyveri	4
	thermotolerans	25
Metchnikowia	*fruiticola*	1
	pulcherrima	1
Meyerozyma	*guilliermondii*	4
Ogatea	*naganishii*	1
Pichia	*fermentans*	3
	galeiformis	12
	guilliermondii	2
	kluyveri	7
	kudriavzevii	9
	manshurica	4
	membranifaciens	5
	mexicana	2
	nakazawae	2

	quercuum	1
Rhodosporidium	*babjevae*	1
	diobovatum	1
Rhodotorula	*mucilaginosa*	1
Saccharomyces	*cerevisiae*	37
	kudriavzevii	1
	paradoxus	8
Schizosaccharomyces	*japonicus*	2
	pombe	4
Starmerella	*bacillaris*	2
	bombicola	3
Torulaspora	*delbrueckii*	24
Wickerhamomyces	*anomalus*	17
Williopsis	*saturnus*	1

662

663

664 **Table 2. Lab-scale fermentation and sensory results for representative heterofermentative**
665 **strains.** The highlighted strains were used in Figures 2 and/or 3.

Strain	Species[a]	Attenuation[b]	Final pH[c]	Sensory notes
WYP39	*L. fermentati*	60%	3.68	Sour, pear, melon, black tea
YH25	*L. fermentati*	60%	3.66	Lactic tart finish
YH26	*L. thermotolerans*	60%	3.35	Very tart, peach, citrus zest
YH27	*L. thermotolerans*	40%	3.42	Tart green apple, clean
YH72	*H. vineae*	75%	3.26	Slightly sour, clean, tart fruit, apple cider, quaffable
YH73	*L. thermotolerans*	55%	3.36	Very sour, berries
YH77	*L. fermentati*	60%	3.55	Tart, clean, pear
YH79	*L. thermotolerans*	50%	3.42	Slightly tart, clean, rounded
YH81	*L. thermotolerans*	55%	3.21	Tart, fruity, citrus, pear, blood orange
YH82	*W. anomalus*	83%	3.24	Slightly sour, fruity, clean, aromatic, tart fruit, reminiscent of perry
YH109	*L. thermotolerans*	55%	3.39	Very sour, clean
YH140	*L. thermotolerans*	50%	3.26	Sour, clean, balanced, tea flavors
YH156	*S. japonicus*	72%	3.88	Sour, fruity, green apple Jolly Rancher

666 [a] The genus abbreviations are: *L.*, *Lachancea*; *H.*, *Hanseniaspora*; *W.*, *Wickerhamomyces*; and *S.*,
667 *Schizosaccharomyces*.

668 [b] The reported attenuation is based on several trial fermentations in various worts.

669 [c] The initial pH was 5.0.

670

671 **Table 3. Large-scale fermentation data for select heterofermentative yeasts.**

Strain	Lag time	FG[b]	Final pH[c]	Flocculation	Sensory
WYP39[a]	6 h	0.099	3.74	Medium	Tart, dry, light pineapple & mango
YH72	14 h	1.000	3.23	Medium	Very sour, stone fruit flavors
YH82	13 h	1.001	3.36	Medium	Very sour, pear, apple, and apricot
YH156	14 h	0.099	3.20	High	Sour, intense stone fruit aroma & flavors

672 [a] WYP39 = *Lachancea fermentati*, YH72 = *Hanseniaspora vineae*, YH82 = *Wickerhamomyces*
673 *anomalus*, and YH156 = *Schizosaccharomyces japonicus*.

674 [b] FG, final gravity; original gravity = 1.046 for all fermentations.

675 [c] The starting pH was 5.35 for all fermentations.

676 **Figure Legends**

677

678 **Figure 1. Evolutionary relationships among the wild strains and a commercially available**
679 **ale yeast.** The D1/D2 rDNA sequences of the indicated strains were aligned, and the
680 phylogenetic relationships among them were drawn as a rooted N-J tree using
681 *Schizosaccharomyces pombe* as the outgroup. From top to bottom, the *S. japonicas* strains are
682 highlighted purple, the *W. anomalus* strains are red, the *H. vineae* strains are green, the *L.*
683 *fermentati* strains are dark blue, and the *L. thermotolerans* strains are light blue. *S. cerevisiae*
684 strain WLP001 is not highlighted and occupies the relative midpoint of the phylogenetic tree.

685 **Figure 2. Colony and cell morphology of the indicated yeast strains.** A) *H. vineae* (YH72), *L.*
686 *fermentati* (WYP39), *L. thermotolerans* (YH140), *S. japonicus* (YH156), and *W. anomalus*
687 (YH82) colony morphology on YPD agar (middle) and WLN agar (right). *S. cerevisiae* strain
688 YH166 was included for comparison. (B-G) Light micrographs of *H. vineae*, *L. fermentati*, *L.*
689 *thermotolerans*, *S. japonicus*, and *W. anomalus* (respectively) taken by differential interference
690 contrast imaging. The black arrow in (F) indicates a probable microhyphae. Magnification =
691 1000x.

692 **Figure 3. Lactic acid production.** A) LASSO assay for lactic acid production by the indicated
693 strains. Cells producing lactic acid develop a dark halo. Images of both the top and bottom of a
694 representative LASSO plate are shown. *S. cerevisiae* WLP001 and LAB (*L. plantarum*) were
695 included as negative and positive controls for lactic acid production, respectively. These results
696 are indicative of three independent experiments using the same strains. B) Multi-well plate assay
697 for lactic acid production. Cell growth medium containing lactic acid turns from gold to green
698 when the enzymatic assay is complete. Multiple strains of *L. thermotolerans, L. fermentati, H.*

699 *vineae*, and LAB were tested, as well as *S. japonicus* YH156 (*Sj*), *W. anomalus* YH82 (*Wa*), *S.*
700 *cerevisiae* WLP001, and *Escherichia coli* DH5α (*E. coli*). WLP001 and *E. coli* served as
701 negative controls for lactic acid production by yeast and bacteria, respectively. *L. plantarum* (left
702 LAB well) and *L. brevis* (right LAB well) were used as positive controls for lactic acid
703 production. C) L-lactate quantification. Typical GC-MS spectra of the lactate standard (solid red
704 line) and isolated lactate from beer samples are shown. HV (solid purple line), WA (solid green
705 line), LF (dashed burnt sienna line), and SJ (dashed black line) represent beers fermented with *H.*
706 *vineae* YH72, *W. anomalus* YH82, *L. fermentati* WYP39, and *S. japonicus* YH156, respectively.
707 "Caul" (solid black line) represents the lactate isolated from Cauldron, a positive control for
708 lactate in beer (See Section 2.6 and (17)).

709 **Figure 4. Comparison of kettle souring, wood-aged souring, and primary souring.** See the
710 text in Section 4.6 for details.

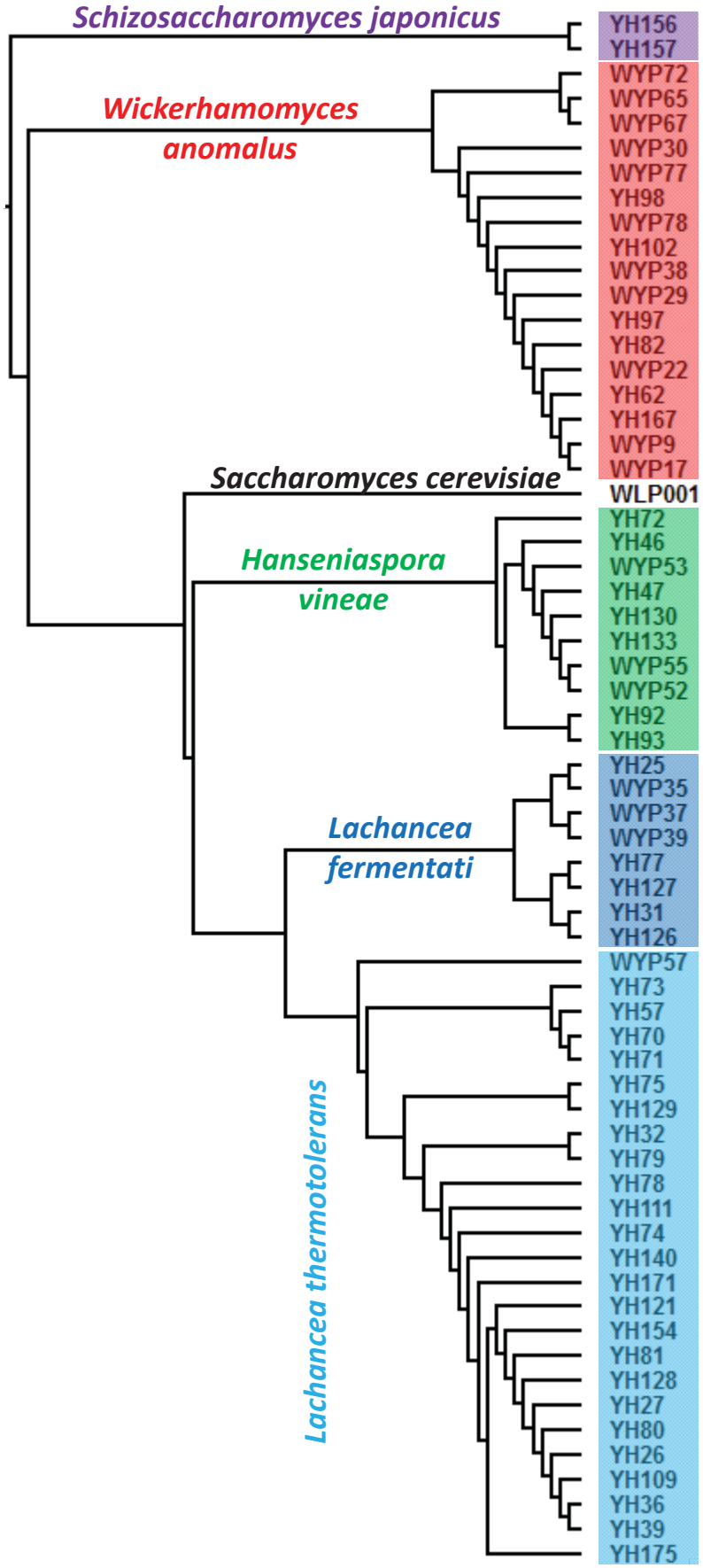


Figure 1

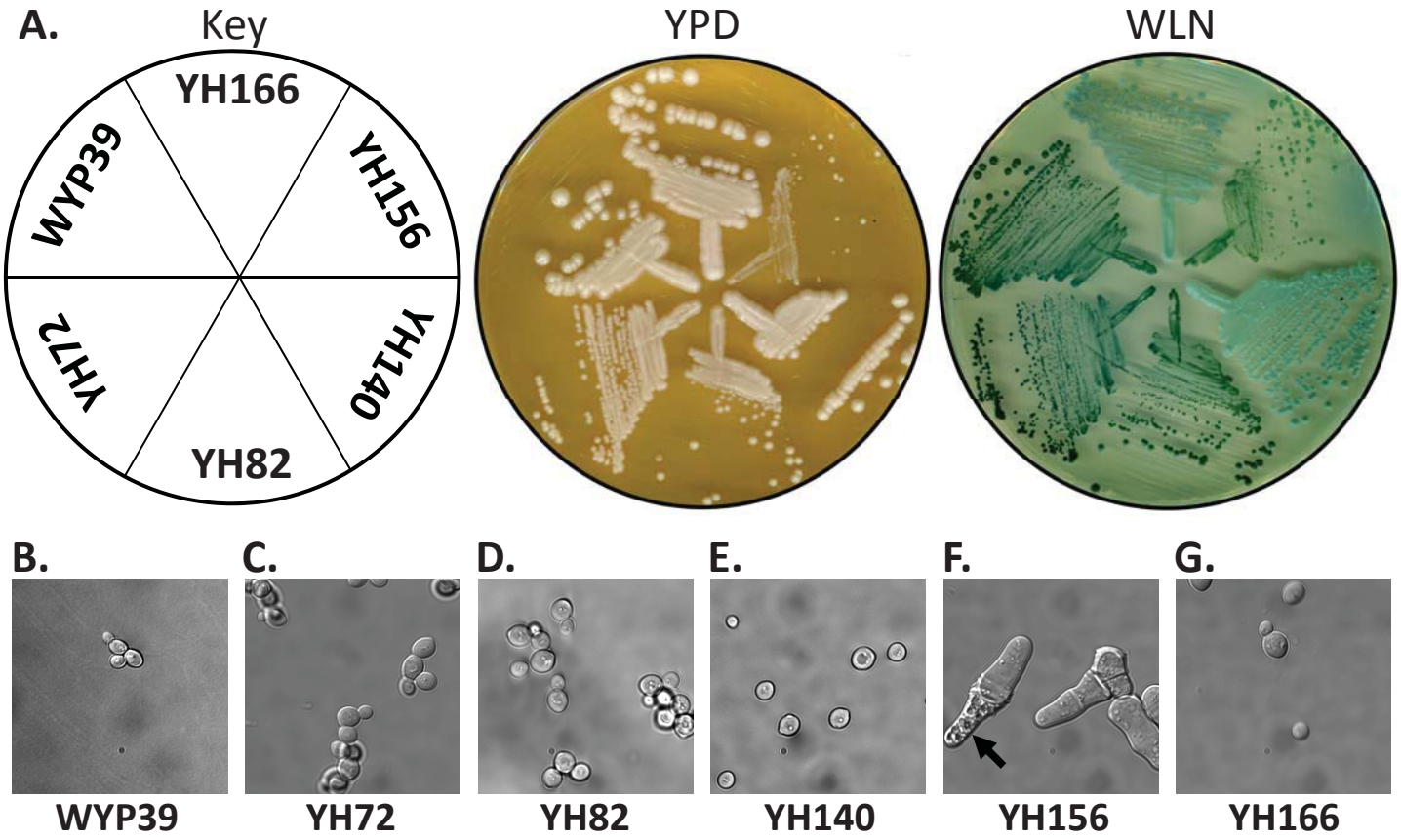
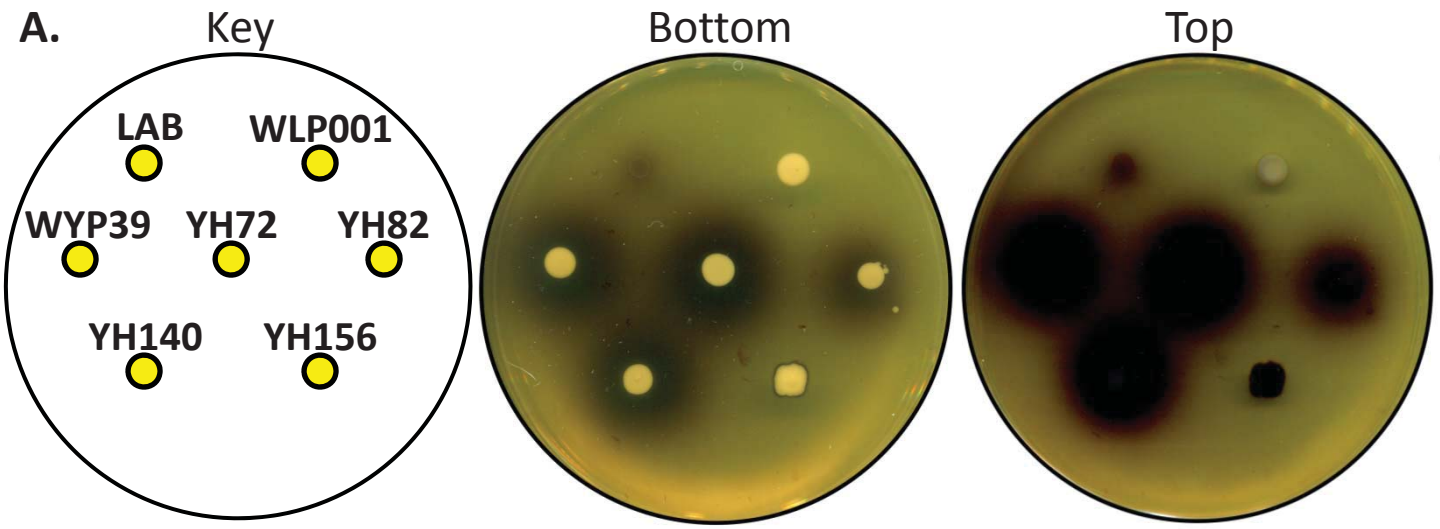
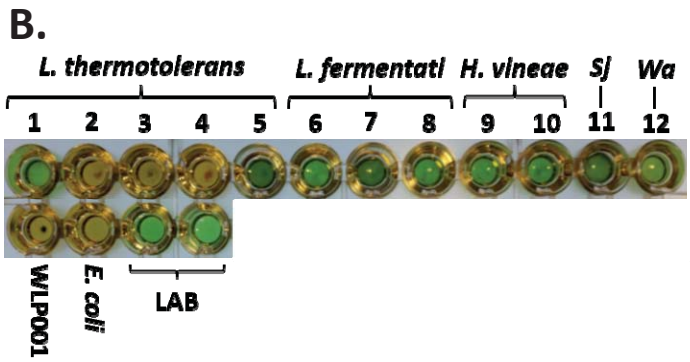


Figure 2





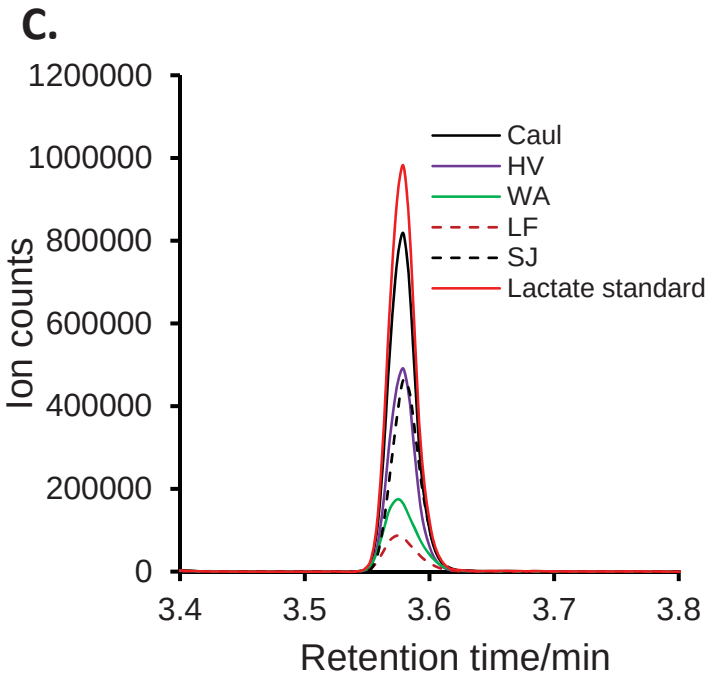


Figure 3

Kettle souring (fast souring)

Wort production

↓

Cool to ~45°C

↓

Inoculate with LAB

↓

Incubate at ~45°C for 48 h

↓

Boil the soured wort

↓

Primary fermentation

Occurs in the brew kettle

Mixed culture souring (long-term souring)

Wort production

↓

Inoculation with yeast (and LAB)

↓

Primary fermentation

↓

Barrel aging (months to years)

Barrels may contain additional yeasts and/or LAB, or a secondary microbe inoculation is performed.

Primary souring

Wort production

↓

Inoculation with heterofermentative yeast

↓

Primary fermentation (2-4 weeks)

⇣

Could be followed by fruit addition, barrel aging, *etc*.

Key: red text denotes when lactic acid production occurs

Figure 4