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

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35 **Abstract**

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

51

52 **Keywords:** yeast, lactic acid, sour beer, heterolactic fermentation

53 **Chemical compounds studied in this article:**

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

55 **Abbreviations:** ABV, alcohol by volume; DIC, differential interference contrast; EtOH, ethanol;
56 FG, final gravity; gDNA, genomic DNA; IBU, international bittering unit; LAB, lactic acid

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57 bacteria; LASSO, lactic acid specific soft-agar overlay; N-J, neighbor-joining; OG, original

58 gravity; WLN, Wallerstein Laboratories nutrient; YPD, yeast extract, peptone, and dextrose

59

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60 **1. Introduction**

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

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

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

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

92

93 **2. Materials and methods**

94 *2.1. Strains, media, and other reagents*

95 *S. cerevisiae* strain WLP001 was purchased from White Labs (San Diego, CA). Wild
96 strains were isolated as described in (5). All yeast strains were routinely grown on yeast extract,
97 peptone, and dextrose (YPD; 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose)
98 plates containing 2% (w/v) agar at 30°C and in YPD liquid culture at 30°C with aeration unless
99 otherwise noted. All strains were stored as 15% (v/v) glycerol stocks at -80°C. Media
100 components were from Fisher Scientific (Pittsburgh, PA, USA) and DOT Scientific (Burnton,
101 MI, USA). All other reagents were of the highest grade commercially available.

102 *2.2. Strain identification and phylogenetic analysis*

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103 To identify wild yeasts at the species level, frozen stocks were streaked onto YPD plates
104 and incubated at 30°C until single colonies formed (18-48 h). Colonies were then picked into
105 microcentrifuge tubes containing 100 µL of lysis solution (0.2 M LiOAc and 1% SDS) and
106 incubated in a 65°C water bath for ≥15 min to lyse the cells. After 300 µL of 100% isopropanol
107 was added to the tubes, they were mixed by vortexing, and the cell debris and genomic DNA
108 (gDNA) were pelleted in a microcentrifuge for 5 min at maximum speed. The supernatant was
109 decanted, and remaining traces were completely removed from the pellets by aspiration. The
110 gDNA was resuspended in 50-100 µL TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA),
111 and a 1-min spin at maximum speed was used to pellet the cell debris to clarify the DNA
112 solution. The variable D1/D2 portion of the eukaryotic 26S rDNA was then amplified by PCR
113 from the gDNA templates using oligos NL1 (GCATATCAATAAGCGGAGGAAAAG) and
114 NL4 (GGTCCGTGTTTCAAGACGG) (11) and the following cycling conditions: 98°C for 5
115 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
116 PCRs were assessed for D1/D2 amplification by running 10% of the reaction volume on 1%
117 (w/v) agarose gels at 100 V (560 bp expected product size). The amplified DNA was then
118 purified using a PCR Purification Kit (Thermo Scientific, Waltham, MA) and quantified using a
119 BioTek Synergy H1 plate reader. The DNA was sequenced by ACGT, Inc. (Wheeling, IL) using
120 primer NL1, and the sequence was used to query the National Center for Biotechnology
121 Information nucleotide database with the Basic Local Alignment Search Tool (BLAST;
122 http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

123 After species identification, the phylogenetic relationships among the isolated strains of
124 *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* were determined by
125 aligning their 26S rDNA sequences using ClustalX (16). The alignments were iterated at each

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126 step but otherwise utilized default parameters. ClustalX was also used to draw and bootstrap
127 neighbor-joining (N-J) phylogenetic trees using 1000 bootstrap trials; the trees were visualized
128 with TreeView v. 1.6.6 software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The
129 *Schizosaccharomyces pombe* rDNA sequence (GenBank accession HE964968) was included in
130 the alignments as the outgroup, and this was used to root the N-J tree in TreeView. WLP001 was
131 included to determine the relatedness of the wild strains to a commercially available ale yeast.
132

133 *2.3. Test fermentations*

134 For laboratory-scale fermentations, select yeast strains were streaked for single colonies
135 onto YPD plates as described above and grown to saturation in 4 mL of YPD liquid medium
136 overnight at 30°C with aeration. The cell count of the starter cultures was approximated by
137 measuring the OD_{660} and converting that value to cells/mL as described at
138 http://www.pangloss.com/seidel/Protocols/ODvsCells.html. In most cases, the saturated
139 overnight cultures reached densities of ~$5x10^8$ cells/mL. These starter cultures were then used to
140 inoculate ~400 mL of blonde ale wort in 500 mL glass bottles capped with drilled rubber
141 stoppers fitted with standard plastic airlocks. The wort was produced by mashing 65.9% Pilsner
142 (2 Row) Bel and 26.9% white wheat malt at 65°C (149°F) for 75 min in the presence of 1 g/bbl
143 $CaCO_3$ and 1.67 g/bbl $CaSO_4$ to yield an original gravity (OG) of 1.044. During the boil, 7.2%
144 glucose was added, as well as Saaz hops to 25 international bittering units (IBUs). The
145 fermentation cultures were incubated at 22.3±0.3°C (~72°F) for 2 weeks. Un-inoculated wort
146 was treated as above to control for wort sterility. Prior to bottling into standard 12-oz brown
147 glass bottles, their final gravity (FG) was measured using a MISCO digital refractometer (Solon,

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148 OH), and pH was measured using an Accumet AB150 pH meter (Fisher Scientific). Bottle
149 conditioning was conducted as in (17) at room temperature for ≥2 weeks.

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

158 *2.4. Lactic acid specific soft-agar overlay (LASSO)*

159 The production of lactic acid by yeast cells was assayed as described in (18). Briefly,
160 cells were grown overnight in liquid YPD medium at 30°C with aeration. Then, 2 µL of each
161 culture was spotted onto YPD10 plates (YPD agar supplemented with glucose to a final
162 concentration of 10% w/v), allowed to absorb, and incubated overnight at 30°C. The plates were
163 then covered with 6.5 mL soft-agar (0.5% agar in 300 mM Tris and 187 mM glutamate, pH 8.3).
164 Upon solidification of the soft agar, a second soft-agar overlay was prepared by mixing 3.2 mL
165 1% agar with 3.2 mL of a staining solution composed of 30 mM Tris/18.75 mM glutamate (pH
166 8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL phenazine methosulfate, 7 U
167 glutamate pyruvate transaminase, and 7 U L[+]-lactate dehydrogenase (all LASSO components
168 were from Sigma-Aldrich, St. Louis, MO). Strains producing lactic acid formed purple halos

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169 within 10 min, the color of which darkened with increasing incubation time at room temperature.
170 Cells not producing lactic acid never formed halos.

171 *2.5. Multi-well lactic acid production assay*

172 Because only a small number of strains can be tested for lactic acid production on a
173 single plate in the LASSO assay, we also adapted it for use in multi-well plates. Briefly,
174 individual strains were grown overnight in 100 µL YPD10 medium in the wells of 96-well plates
175 at 30°C with aeration in a BioTek Synergy H1 plate reader. To avoid evaporation of the medium,
176 50 µL mineral oil was used to overlay each well. Then, 100 µL of staining solution (30 mM
177 Tris/18.75 mM glutamate (pH 8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL
178 phenazine methosulfate, 1 U/mL glutamate pyruvate transaminase, and 1 U/mL L[+]-lactate
179 dehydrogenase) was added to each well and mixed by agitation in the plate reader. The reaction
180 proceeded for ≥ 10 min at room temperature, and the presence of lactic acid was indicated by the
181 gold colored solution turning green (and eventually blue with extended incubation).

182 *2.6. Beer sensory analysis*

183 Sensory analysis was performed by 10 volunteers with various levels of experience, from
184 neophytes to those with Beer Judge Certification Program (19) training. In all cases, the sensory
185 descriptors (*e.g.*, Barnyard, Bitter, Body, Drinkability, Dry, Estery, Harsh, Hoppy, Malty,
186 Papery, Sour, Sulfury, and Sweet in Supplemental Fig. S3) were defined and described to the
187 participants, and commercial calibrations beers were used as examples of sour (Cauldron;
188 Upland Brewing Company, Bloomington, IN, USA) and clean beers (Dragonfly IPA; Upland
189 Brewing Company). Then, chilled samples of the experimental beers were provided to the
190 volunteers, and they were instructed to write down aroma and flavor descriptions, as well as to

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191 rank each of the Supplemental Figure S3 flavor descriptors on a 1-10 scale. These analyses were
192 performed in a blinded manner, with none of the participants knowing which yeast strains
193 fermented the beers. After individual assessments, the group discussed the sensory attributes of
194 each beer to come to a consensus on the best descriptions of aroma and flavor, which are
195 reported throughout this work.

196 *2.7. Gas chromatography-mass spectrometry (GC-MS) analysis of lactic acid*

197 To determine the concentration of lactic acid in beer samples, 20 µL of beer and lactate
198 standards were individually added to 900 µL of 90% methanol containing 1.25 µg/mL succinic-
199 d4 acid in 1.5-mL microfuge tubes. The samples were vortexed for 10 s, incubated for 1 h at -
200 20ºC, and centrifuged at 20,000 x g for 5 min at 4ºC. The cleared supernatants were transferred
201 to 1.5-mL tubes and dried overnight using a vacuum centrifuge (Savant). Subsequent
202 derivatization and GC-MS analysis were performed as previously described (20). Lactate
203 concentration was determined using a standard curve plotted from the analyzed lactate standards
204 of known concentration.

205

206 **3. Results**

207 *3.1. Discovery of five heterofermentative yeast species*

208 We previously reported an initial description of our bio-prospecting and characterization
209 of 100 wild yeasts for use in the brewing industry (5). We have increased our collection to 284
210 strains from 54 different species in 26 genera (Supplemental Table 1). To determine the relative
211 usefulness of these strains in beer brewing, small laboratory-scale beer fermentations were

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212 performed for each isolate. During our sensory analyses of the resultant beers, we noted that
213 many of the strains were producing beers that were characterized as tart or sour (Table 2), akin to
214 styles that are produced with the aid of LAB (21). Initially, we hypothesized that these
215 experimental beers may have been contaminated by LAB. However, when we phylogenetically
216 grouped these strains, we found that they were all members of five species in four genera: *H.*
217 *vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* (Supplemental Table 1
218 and Fig. 1). Because these strains were not randomly distributed throughout the many species in
219 our collection, we hypothesized that the yeasts may be producing the lactic acid themselves. To
220 determine if this apparent heterofermentative activity was specific to evolutionarily closely
221 related yeasts, we aligned the sequences of the D1/D2 variable region of their ribosomal DNA
222 and plotted a phylogenetic tree. As shown in Figure 1 and Figure S1, three of the species (*H.*
223 *vineae*, *L. fermentati*, and *L. thermotolerans*) are closely related to ale yeast (WLP001), but the
224 other two (*S. japonicus*, and *W. anomalus*) form more distinct clades, with the divergence
225 between budding yeasts such as *S. cerevisiae* (*e.g.*, WLP001) and fission yeasts such as *S.*
226 *japonicus* (*e.g.*, YH156) occurring approximately 1 billion years ago (22).

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

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235 *3.4. Strains* L. fermentati *WYP39,* H. vineae *YH72,* W. anomalus *YH82,* L. thermotolerans
236 *YH140, and* S. japonicus *YH156 produce lactic acid*

237 We further sought to determine if strains *L. fermentati* WYP39, *H. vineae* YH72, *W.*
238 *anomalus* YH82, *L. thermotolerans* YH140, and *S. japonicus* YH156 were truly producing lactic
239 acid during fermentation, rather than one or more other secondary metabolites that yield a
240 tart/sour flavor (23). Using the LASSO assay for lactic acid production by yeast (18), we found
241 that all five strains produced lactic acid (denoted by dark halos in Fig. 2A), similar to the
242 *Lactobacillus plantarum* positive control. In contrast, the *S. cerevisiae* WLP001 negative control
243 failed to generate a halo. Because the LASSO assay can only be used for a limited number of
244 strains on a single plate, we adapted it into a multi-well plate assay (Fig. 2B). Here, lactic acid
245 production is evident by the golden-colored assay medium turning green, as indicated by the
246 LAB controls. Again, WLP001 failed to generate detectable lactic acid, as did a common
247 research strain of *E. coli*. However, multiple tested isolates of *L. thermotolerans*, *L. fermentati*,
248 *H. vineae*, *S. japonicus*, and *W. anomalus* did test positive for lactic acid production. Some
249 individual *L. thermotolerans* strains failed to generate lactic acid (wells 2 and 4) or did so slowly,
250 as certain wells were just beginning to turn green (well 3) when the image in Figure 2B was
251 acquired. These results correspond with sensory analysis of beers fermented with the various *L.*
252 *thermotolerans* strains, which ranged from not sour to very tart (Table 2 and data not shown).

253 *3.4. Analysis of beers fermented with lactic acid-producing yeasts*

254 To monitor the activities of *L. fermentati* WYP39, *H. vineae* YH72, *W. anomalus* YH82,
255 and *S. japonicus* YH156 in larger-scale fermentations, we inoculated these strains into glass
256 carboys containing 19 L (5 gal) each of an identical blonde wort. Because the beer brewing

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257 capabilities of a *L. thermotolerans* strain were recently described (14), we omitted strain *L.*
258 *thermotolerans* YH140 from these assays to avoid generating redundant data. All of the strains
259 had short lag times (*i.e.*, the approximate time from inoculation to the first visible signs of
260 fermentation) ranging from 6-14 h (Table 3). These lag times to fermentation were similar to that
261 of WLP001 inoculated in a similar blonde wort (~12 h, Table 3). However, the kinetics of the
262 full fermentation differed for each lactic acid-producing yeast. *L. fermentati* WYP39 had the
263 shortest lag time and fermented rapidly for 2 weeks, at which point it slowed considerably and
264 required an additional 2 weeks to reach a final gravity of 0.099 (Table 3). *H. vineae* YH72 was a
265 slow and steady fermenter, attaining a terminal gravity of 1.000 after 3 weeks. *W. anomalus*
266 YH82 was similar, but required a full 4 weeks to reach a final gravity of 1.001.

267 The *S. japonicus* YH156 strain displayed the most variant fermentative characteristics.
268 After reaching a vigorous state of fermentation at 14 h (Table 3), popcorn-like clusters of cells
269 formed and floated around within the fermenter (Supplemental Fig. 2). Eventually, they settled
270 into a mountainous pile against one side of the carboy before compacting down into a yeast
271 slurry with a typical appearance on the bottom of the fermenter. The fermentation reached a final
272 gravity of 0.099 approximately 27 days after inoculation. The final pH of each beer was recorded
273 and varied from a low of 3.20 to a high of 3.74. Sensory analyses of each beer were conducted
274 (Supplemental Fig. 3), and the tasting notes are discussed in Sections 4.1-4.5 below.

275 We also quantified the concentration of L-lactic acid present in each beer by GC-MS
276 (Fig. 2C and S4). We used Cauldron, a commercially available sour beer made by mixed
277 fermentation of yeast and LAB (17), as a positive control for lactic acid production; it contained
278 100.54 mM lactate. Based on a lactate standard curve, *L. fermentati* WYP39, *H. vineae* YH72,

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279 *W. anomalus* YH82, and *S. japonicus* YH156 produced 10.02, 35.69, 29.05, and 50.09 mM
280 lactate, respectively.

281 The *S. japonicus* YH156 results contrast with those from the LASSO assay in Figure 2A,
282 where *S. japonicus* YH156 displayed the least evidence of lactic acid production. Because the
283 lactic acid production occurred in the presence of oxygen in the LASSO assay, this may indicate
284 that *S. japonicus* YH156 is Crabtree negative or only weakly Crabtree positive, *i.e.*, requiring
285 anaerobic conditions for fermentation (see (24) and references therein). It should also be noted
286 that the beers analyzed by GC-MS were fermented in a brewery that utilizes LAB, and thus, it
287 remains a formal possibility that they may have been inadvertently contaminated by other
288 organisms that can generate lactic acid. However, the results in Figures 2A and 2B are from pure
289 cultures (as judged by post-fermentation plating, microscopy, and PCR analyses of the yeast
290 slurry for LAB contamination; Supplemental Fig. 5 and data not shown) of *L. fermentati*
291 WYP39, *H. vineae* YH72, *W. anomalus* YH82, and *S. japonicus* YH156. These cultures also still
292 acidified beer during fermentation in the presence of antibiotics or 75 IBU wort in a laboratory
293 setting (Supplemental Table 2), strongly suggesting that LAB contamination was not the source
294 of the lactic acid.

295 **4. Discussion**

296 The next phase in the “local” movement in the beer industry will be the isolation and use
297 of local yeast strains in brewing. Indeed, in the U.S., nearly all commercially available ale and
298 lager strains are of European origin, so no American beer will ever truly be local without the
299 inclusion of New World yeast. Here, we detailed the initial characterization of nearly 300 local
300 strains for use in fermentation. Within this strain bank, we uncovered five species that generate

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301 lactic acid and ethanol during primary fermentation and suggest that they can be used in a novel,
302 LAB-free beer souring method (see Section 4.6. below).

303 *4.1.* H. vineae

304 To our knowledge, this is the first report of pure cultures of *H. vineae* being used to
305 ferment beer. As the species name suggests, *H. vineae* is typically associated with wine, where it
306 has previously been investigated alone and in combination with *S. cerevisiae* for grape must
307 fermentation (25-27). The strains previously tested are notable for the production of high levels
308 of 2-phenylethyl acetate, which is an aromatic compound that lends floral, fruity, and/or honey-
309 like notes to wine (26). Indeed, in our trial fermentations with *H. vineae* YH72, we often noted
310 fruity aromas and flavors (Tables 2 and 3).

311 Although yeasts in the *Hansenia* genus are the predominant species found on grapes (28-
312 30), they are also found elsewhere (reviewed in (31)). Strain *H. vineae* YH72 was isolated from a
313 white ash tree (*Fraxinus americana*) in southwestern Pennsylvania (Table 2) and was the only
314 *Hanseniaspora* isolate to consistently produce tart beer (see Supplemental Table 3). It ferments
315 slowly relative to typical commercially available ale yeasts, but reached high levels of
316 attenuation after only two weeks (Table 2) and further attenuated with additional fermentation
317 time (Table 3). The beers produced by short fermentations with *H. vineae* YH72 were slightly
318 sour but clean and highly drinkable, with notes of apple cider. Longer fermentation yielded very
319 sour beer with a pH (3.23) and acidic bite reminiscent of beers produced with LAB, as well as
320 stone fruit notes (Table 3, Supplemental Fig. 3). Currently, we are further characterizing the
321 fermentative capabilities and acid production of *H. vineae* YH72 and other *Hansenia* species.

322 *4.2.* L. fermentati

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323 Very little is known about *L. fermentati*, especially with regard to beverage fermentation.
324 Industrially, this species of yeast has been found in fermented (wine (32), cachaça (33), and
325 water kefir (34)) and non-fermented beverages (coconut water and fruit juices (35)). However,
326 its effects on the sensory characteristics of these beverages are largely unknown. As with *H.*
327 *vineae* YH72 above, this also appears to be the first report of beer fermented with pure cultures
328 of *L. fermentati*. Our laboratory-scale test fermentations indicated that strain WYP39 displayed
329 decent wort attenuation for a wild strain (60%, Table 2), and longer fermentation in a larger-
330 scale fermentation yielded a dry product (Table 3). The final pH was modest compared to other
331 sour beers (Table 3), creating a flavor that was more tart than sour, but this was accentuated by
332 light pineapple and mango flavors. There are many species in the *Lachancea* genus (36). Based
333 on the desirable brewing characteristics of *L. fermentati* and *L. thermotolerans* (discussed below
334 in Section 4.3. and in Domizio *et al*. (14)), it will be interesting to assess the activities of these
335 other species during beer fermentation.

336 *4.3.* L. thermotolerans

337 Various strains of *L. theromtolerans* have been studied for their effects on wine
338 fermentation (reviewed in (29)), though usually in co-fermentations with *S. cerevisiae* (*e.g.*,
339 (37,38). Recently, *L. thermotolerans* strain Lt101 was shown to be a viable yeast for beer
340 production (14), and the same group also found that three *L. thermotolerans* strains including
341 Lt101 produce lactic acid during fermentation. Although this is similar to our observations
342 (Table 2 and data not shown), the strains investigated by Domizio *et al*. (14) only decreased the
343 pH of wort from 5.66 to 4.28-3.77 during fermentation. Most of their experiments yielded final
344 pH values in the 4.17-4.3 range, however, which is similar to the pH decrease caused by *S.*
345 *cerevisiae* UCD 915. Our *L. thermotolerans* isolates that produced noticeably tart beers reached

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346 terminal pH values of ~3.35 (Table 2 and data not shown). These discrepancies are likely due to
347 differences in the experimental set ups, as well as strain-to-strain variability, which is discussed
348 in Section 5 below.

349 *4.4.* S. japonicus

350 *S. japonicus* is closely related to *S. pombe*, which was originally isolated from African
351 millet beer (reviewed in (39)). *S. japonicus* itself was first isolated from strawberries in Japan
352 (40) and is associated with indigenous fermented beverages (*e.g.*, kaffir beer, plantain beer, palm
353 wine, sugar cane wine, and sake) around the world (41), wine production (42), and was isolated
354 from spontaneously fermented beer in North Carolina (43). However, no characterization of that
355 beer is available. Thus, this is the first rigorous report of *S. japonicus* used for primary
356 fermentation of beer. The attenuation levels of strain *S. japonicus* YH156 were excellent in both
357 laboratory- and large-scale fermentations (Table 2 and 3), and the aroma and flavor profiles of
358 these beers included common descriptors of sour, fruity, and stone fruit. Individual tasters
359 identified green apple Jolly Rancher, tart apple, pear, pineapple, and peach notes.

360 *4.5.* W. anomalus

361 *W. anomalus*, previously known as *Saccharomyces anomalus* (44), has multiple roles in
362 the biotechnology, agriculture, and food production fields. It is often found in association with
363 grain and is especially useful to inhibit storage molds during malting (45). Concerning beverage
364 fermentation, *W. anomalus* is generally referred to as a beer spoilage organism (46), but it is also
365 necessary for cocoa and coffee bean fermentation (47). Although *W. anomalus* has been
366 investigated for its use in apple wine and hard cider production (48,49), the only work involving
367 beer has focused on the spoilage properties of this species (46). As with *S. japonicus* YH156

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368 above, the YH82 strain of *W. anomalus* yielded excellent attenuation at both fermentation scales
369 tested (Tables 2 and 3). This strain produced a less intense sour character than others, but the
370 beer was characterized as clean, aromatic, and fruity with notes of pear, apple, and apricot.

371 *4.6. Primary souring*

372 There are two general methods by which sour beers are produced: kettle souring and
373 mixed culture fermentation (Fig. 3) (21). Kettle souring is the more rapid and modern technique.
374 This method affords brewers tight control over acid production (souring can be stopped at any
375 time via boiling) and is less time consuming that mixed culture fermentation (below), but it also
376 has inherent weaknesses. First, the entire souring process occurs in the brew kettle, so it prevents
377 additional wort production in that vessel. Indeed, kettle souring is often relegated to weekends
378 when small breweries are otherwise not in production mode. Second, boiling the wort after it has
379 been soured drives off volatile aromatics that may also have been produced during souring,
380 yielding beers that are described and criticized as lacking in depth and character. To combat
381 some of these sensory downsides, some brewers are now barrel aging kettle sours to impart oak
382 complexity to the final beers.

383 In contrast, souring by mixed culture fermentation is the more traditional method. It
384 produces more complex and nuanced flavors in beer than kettle souring, but it suffers from a
385 huge time lag from wort production to the final beer and requires a large space dedicated to
386 housing the barrels. Further, the souring organisms are usually alive throughout the beer
387 production and packaging processes, so separate equipment is usually necessary to prevent the
388 accidental contamination of non-sour (*i.e.*, clean) beers.

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389 Here, we propose a third paradigm for sour beer production that we call primary souring.
390 In this method, the wort is inoculated with a yeast capable of heterolactic fermentation rather
391 than *S. cerevisiae*, and souring occurs during primary fermentation in the absence of LAB (Fig.
392 3). The yeast strains described in Tables 2 and 3 and in the text above did not display rapid
393 fermentation kinetics like commercially available ale yeasts, but they still completed
394 fermentation within a month, displaying excellent levels of attenuation and medium-to-high
395 flocculation (Table 3). Further, the sensory profiles of the beers were superior to kettle soured
396 beer, displaying both lactic tartness and fruity aromatic and flavor notes. Compared to the sour
397 production methods above, primary souring is beneficial in that it frees up the brew kettle and
398 does not require lengthy aging in barrels, though oak aging is a possibility after primary
399 fermentation (Fig. 3). Perhaps most alluringly, primary souring does not require the introduction
400 of bacteria into the brewery, and preliminary tests suggest that *H. vineae*, *L. fermentati*, *L.*
401 *thermotolerans*, *S. japonicus*, and *W. anomalus* can be eliminated as easily as *S. cerevisiae* from
402 brewing equipment using standard clean-in-place protocols. As is typical of yeasts, these species
403 are also hop tolerant (Supplemental Table 2), enabling more liberal use of hops in wort
404 production for sour beers. However, it should be noted that yeast growth can be inhibited by hop
405 iso-α-acids in acidic milieus (50), so the absolute levels of hop tolerance will likely vary by
406 strain and the desired pH of the sour beer.

407

408 **5. Conclusions and Outlook**

409 We set out to isolate and characterize new yeasts for use in beer fermentation. Here, we
410 highlight the discovery of a set of yeast species that produce both lactic acid and ethanol during

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411 primary fermentation. It is unclear how widespread this heterolactic fermentation phenotype is
412 among ethanol-tolerant yeasts, but its presence in the fission yeast *S. japonicus* and budding
413 yeasts like *H. vineae*, which are separated by ~1 billion years of evolution (22), may indicate that
414 heterolactic fermentation is an ancient and conserved metabolic process among single-celled
415 fungi. Arguing against this hypothesis is the lack of detectable lactic acid production by related
416 strains of the same species, *e.g.*, differences among *L. thermotolerans* isolates ((14) and data not
417 shown). Whole genome sequencing and/or transcriptomic analysis of lactic acid-producing yeast
418 during fermentation is needed to determine how the heterolactic fermentation occurs. Regardless,
419 it is our hope that the strains described above and the primary souring process put forth here will
420 add strength to the already growing sour beer movement in the U.S. and abroad.

421

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428

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558 **Tables**

559 **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

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	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

560

561

562 **Table 2. Lab-scale fermentation and sensory results for representative heterofermentative strains.** The highlighted strains were
563 used in Figure 2.

Strain	Species[a]	Place of origin	Collected from	Attenuation[b]	Final pH[c]	Sensory notes
WYP39	*L. fermentati*	Evansville, IN	Birch bark	60%	3.68	Sour, pear, melon, black tea
YH25	*L. fermentati*	New River Gorge, WV	Red oak bark	60%	3.66	Lactic tart finish
YH26	*L. thermotolerans*	New River Gorge, WV	Tulip poplar bark	60%	3.35	Very tart, peach, citrus zest
YH27	*L. thermotolerans*	New River Gorge, WV	Red oak bark	40%	3.42	Tart green apple, clean
YH72	*H. vineae*	New Kensington, PA	Ash bark	75%	3.26	Slightly sour, clean, tart fruit, apple cider, quaffable
YH73	*L. thermotolerans*	New Kensington, PA	Mulberries	55%	3.36	Very sour, berries
YH77	*L. fermentati*	Princeton, NJ	Red oak bark	60%	3.55	Tart, clean, pear
YH79	*L. thermotolerans*	Holmdel, NJ	Red oak bark	50%	3.42	Slightly tart, clean, rounded
YH81	*L. thermotolerans*	Holmdel, NJ	White oak bark	55%	3.21	Tart, fruity, citrus, pear, blood orange
YH82	*W. anomalus*	Sarver, PA	Shumard oak bark	83%	3.24	Slightly sour, fruity, clean, aromatic, tart fruit, reminiscent of perry
YH109	*L. thermotolerans*	Indianapolis, IN	Bell pepper	55%	3.39	Very sour, clean
YH140	*L. thermotolerans*	Bloomington, IN	Shagbark hickory bark	50%	3.26	Sour, clean, balanced, tea flavors
YH156	*S. japonicus*	Northeastern PA	Oak bark	72%	3.88	Sour, fruity, green apple Jolly Rancher

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

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

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566 [c] The initial pH was 5.0.

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567 **Table 3. Large-scale fermentation data for select heterofermentative yeasts and the**
568 **WLP001 control.**

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
WLP001	12 h	1.010	4.35	Medium	Neutral, clean, slightly fruity

569 [a] WYP39 = *Lachancea fermentati*, YH72 = *Hanseniaspora vineae*, YH82 = *Wickerhamomyces*
570 *anomalus*, YH156 = *Schizosaccharomyces japonicus*, and WLP001 = *Saccharomyces cerevisiae*.

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

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

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573 **Figure Legends**

574

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

582 **Figure 2. Lactic acid production.** A) LASSO assay for lactic acid production by the indicated
583 strains. Cells producing lactic acid develop a dark halo. Images of both the top and bottom of a
584 representative LASSO plate are shown. *S. cerevisiae* WLP001 and LAB (*L. plantarum*) were
585 included as negative and positive controls for lactic acid production, respectively. These results
586 are indicative of three independent experiments using the same strains. B) Multi-well plate assay
587 for lactic acid production. Cell growth medium containing lactic acid turns from gold to green
588 when the enzymatic assay is complete. Multiple strains of *L. thermotolerans, L. fermentati, H.*
589 *vineae*, and LAB were tested, as well as *S. japonicus* YH156 (*Sj*), *W. anomalus* YH82 (*Wa*), *S.*
590 *cerevisiae* WLP001, and *Escherichia coli* DH5α (*E. coli*). WLP001 and *E. coli* served as
591 negative controls for lactic acid production by yeast and bacteria, respectively. *L. plantarum* (left
592 LAB well) and *L. brevis* (right LAB well) were used as positive controls for lactic acid
593 production. C) L-lactate quantification. Typical GC-MS spectra of the lactate standard (solid red
594 line) and isolated lactate from beer samples are shown. HV (solid purple line), WA (solid green
595 line), LF (dashed burnt sienna line), and SJ (dashed black line) represent beers fermented with *H.*

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596 *vineae* YH72, *W. anomalus* YH82, *L. fermentati* WYP39, and *S. japonicus* YH156, respectively.
597 "Caul" (solid black line) represents the lactate isolated from Cauldron, a positive control for
598 lactate in beer (See Section 2.6 and (17)).

599 **Figure 3. Comparison of kettle souring, wood-aged souring, and primary souring.** Left)
600 During kettle souring, unhopped or lightly hopped wort is produced in a brew kettle as normal,
601 but then it is only partially cooled to approximately 40-45°C. This temperature favors the growth
602 of LAB, which can be introduced by inoculation with pure cultures or the addition of grain. The
603 LAB then sour the wort in the brew kettle to the desired pH, which usually occurs over the
604 course of 24-48 h. The soured wort in the kettle is ultimately boiled a second time to kill the
605 LAB, and hops can be introduced at this point. The wort is then transferred to a fermenter and
606 (typically) inoculated with *S. cerevisiae* for primary fermentation. Middle) During mixed culture
607 souring, lightly hopped wort is produced in the brew kettle and transferred to a fermenter. There,
608 it can be inoculated with *S. cerevisiae* for primary fermentation. In some cases, LAB are added
609 prior to or concurrently with *S. cerevisiae* (or *Brettanomyces* spp. in 100% "Brett" beers). If the
610 LAB are added at this stage, souring begins during primary fermentation. After the yeast has
611 attenuated the beer to the desired level, it is then barrel aged for months or years until it attains a
612 low pH and complex flavor profile. Barrel aging is another stage at which LAB and
613 *Brettanomyces* spp. can be added (either resident in the barrels or as pure inocula) to induce
614 souring. Right) Primary souring: see the text in Section 4.6 for details.

Schizosaccharomyces japonicus
YH156
YH157
Wickerhamomyces anomalus
WYP72
WYP65
WYP67
WYP30
WYP77
YH98
WYP78
YH102
WYP38
WYP29
YH97
YH82
WYP22
YH62
YH167
WYP9
WYP17
Saccharomyces cerevisiae
WLP001
Hanseniaspora vineae
YH72
YH46
WYP53
YH47
YH130
YH133
WYP55
WYP52
YH92
YH93
Lachancea fermentati
YH25
WYP35
WYP37
WYP39
YH77
YH127
YH31
YH126
Lachancea thermotolerans
WYP57
YH73
YH57
YH70
YH71
YH75
YH129
YH32
YH79
YH78
YH111
YH74
YH140
YH171
YH121
YH154
YH81
YH128
YH27
YH80
YH26
YH109
YH36
YH39
YH175

A.

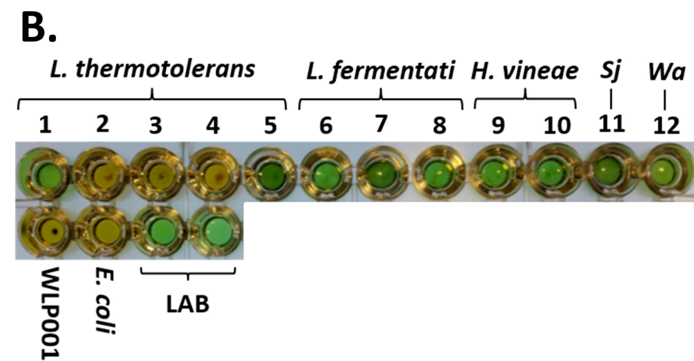
Key

LAB
WLP001
WYP39
YH72
YH82
YH140
YH156

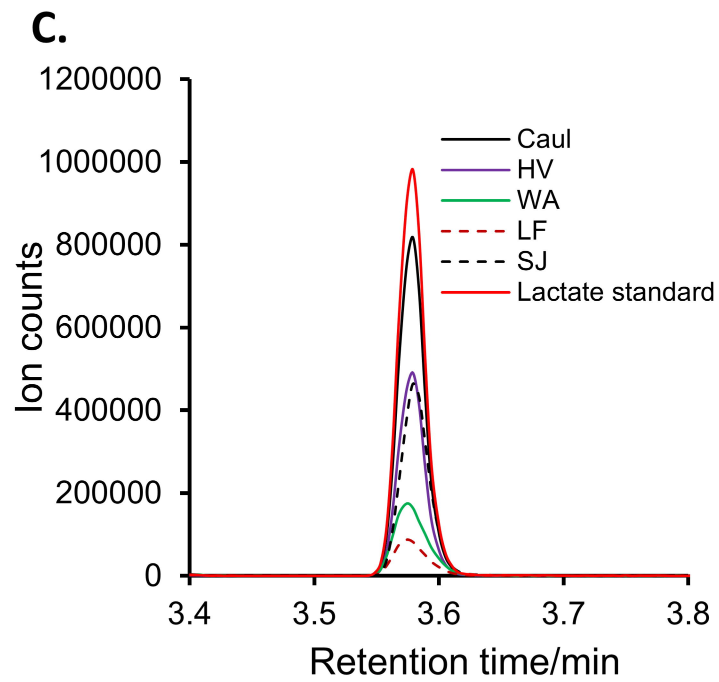
Bottom

Top

B.



C.



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