1      **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:** yeast, lactic acid, sour beer, heterolactic fermentation

**Chemical compounds studied in this article:**

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

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

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

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.

4

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. Wallerstein Laboratories nutrient (WLN) agar contained 4 g/L yeast extract, 5

100     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

101     $MgSO_4$, 2.5 mg/L $FeCl_3$, 2.5 mg/L $MnSO_4$, 22 mg/L bromocresol green, and 15 g/L agar. All

102     strains were stored as 15% (v/v) glycerol stocks at -80°C. Media components were from Fisher

5

103    Scientific (Pittsburgh, PA, USA) and DOT Scientific (Burnton, MI, USA). All other reagents

104    were of the highest grade commercially available.

105    *2.2. Strain identification and phylogenetic analysis*

106         To identify wild yeasts at the species level, frozen stocks were streaked onto YPD plates

107    and incubated at 30°C until single colonies formed (18-48 h). Colonies were then picked into

108    microcentrifuge tubes containing 100 µL of lysis solution (0.2 M LiOAc and 1% SDS) and

109    incubated in a 65°C water bath for ≥15 min to lyse the cells. After 300 µL of 100% isopropanol

110    was added to the tubes, they were mixed by vortexing, and the cell debris and genomic DNA

111    (gDNA) were pelleted in a microcentrifuge for 5 min at maximum speed. The supernatant was

112    decanted, and remaining traces were completely removed from the pellets by aspiration. The

113    gDNA was resuspended in 50-100 µL TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA),

114    and a 1-min spin at maximum speed was used to pellet the cell debris to clarify the DNA

115    solution. The variable D1/D2 portion of the eukaryotic 26S rDNA was then amplified by PCR

116    from the gDNA templates using oligos NL1 (GCATATCAATAAGCGGAGGAAAAG) and

117    NL4 (GGTCCGTGTTTCAAGACGG) (11) and the following cycling conditions: 98°C for 5

118    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

119    PCRs were assessed for D1/D2 amplification by running 10% of the reaction volume on 1%

120    (w/v) agarose gels at 100 V (560 bp expected product size). The amplified DNA was then

121    purified using a PCR Purification Kit (Thermo Scientific, Waltham, MA) and quantified using a

122    BioTek Synergy H1 plate reader. The DNA was sequenced by ACGT, Inc. (Wheeling, IL) using

123    primer NL1, and the sequence was used to query the National Center for Biotechnology

124    Information nucleotide database with the Basic Local Alignment Search Tool (BLAST;

125    http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

126        After species identification, the phylogenetic relationships among the isolated strains of

127    *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* were determined by

128    aligning their 26S rDNA sequences using ClustalX (16). The alignments were iterated at each

129    step but otherwise utilized default parameters. ClustalX was also used to draw and bootstrap

130    neighbor-joining (N-J) phylogenetic trees using 1000 bootstrap trials; the trees were visualized

131    with TreeView v. 1.6.6 software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The

132    *Schizosaccharomyces pombe* rDNA sequence (GenBank accession HE964968) was included in

133    the alignments as the outgroup, and this was used to root the N-J tree in TreeView. WLP001 was

134    included to determine the relatedness of the wild strains to a commercially available ale yeast.

135

136    *2.3. Test fermentations*

137        For laboratory-scale fermentations, select yeast strains were streaked for single colonies

138    onto YPD plates as described above and grown to saturation in 4 mL of YPD liquid medium

139    overnight at 30°C with aeration. The cell count of the starter cultures was approximated by

140    measuring the $OD_{660}$ and converting that value to cells/mL as described at

141    http://www.pangloss.com/seidel/Protocols/ODvsCells.html. In most cases, the saturated

142    overnight cultures reached densities of ~$5x10^8$ cells/mL. These starter cultures were then used to

143    inoculate ~400 mL of blonde ale wort in 500 mL glass bottles capped with drilled rubber

144    stoppers fitted with standard plastic airlocks. The wort was produced by mashing 65.9% Pilsner

145    (2 Row) Bel and 26.9% white wheat malt at 65°C (149°F) for 75 min in the presence of 1 g/bbl

146    $CaCO_3$ and 1.67 g/bbl $CaSO_4$ to yield an original gravity (OG) of 1.044. During the boil, 7.2%

147    glucose was added, as well as Saaz hops to 25 international bittering units (IBUs). The

148    fermentation cultures were incubated at 22.3±0.3°C (~72°F) for 2 weeks. Un-inoculated wort

149    was treated as above to control for wort sterility. Prior to bottling into standard 12-oz brown

150    glass bottles, their final gravity (FG) was measured using a MISCO digital refractometer (Solon,

151    OH), and pH was measured using an Accumet AB150 pH meter (Fisher Scientific). Bottle

152    conditioning was conducted as in (17) at room temperature for ≥2 weeks.

153           Small-batch fermentations were performed at Mainiacal Brewing in Bangor, ME. To

154    produce the test wort, 93.4% two-row base malt and 6.6% carapils were mashed at 66.7°C

155    (152°F) to yield an OG of 1.046. During the boil, Loral hops were added to a final concentration

156    of 5.3 IBUs. The wort was then chilled and split into 5-gal portions in separate carboys.

157    Approximately $1x10^{11}$ cells of the indicated yeast strains were used to inoculate the carboys and

158    allowed to ferment under anaerobic conditions at 21.7°C (71°F) for 1 month. Gravity

159    measurements were taken both with a hydrometer and refractometer by standard methods. The

160    final pH was recorded prior to bottling and bottle conditioning as above.

161    *2.4. Yeast morphology analysis*

162           To determine colony morphology, the yeast strains were first grown with aeration

163    overnight at 30°C in liquid YPD medium. Small volumes were then spotted onto YPD and WLN

164    plates and streaked for single colonies. The plates were incubated at 30°C for ~48 h before

165    imaging with an Epson V37 flatbed scanner. The same liquid cultures were sampled for

166    microscopic analysis in the Indiana University Light Microscopy Imaging Center. Briefly, ~5 μL

167    samples of cell culture were spotted onto 25 x 75 mm (1.0 mm thick) glass slides and covered

168    with a 22 x 22 mm no. 1.5 cover slip. The live cells were then observed by differential

169    interference contrast under oil immersion at 1000x magnification using a Nikon NiE microscope.

170    Digital images of the fields of view were collected using Nikon Elements software.

171    *2.5. Lactic acid specific soft-agar overlay (LASSO)*

172         The production of lactic acid by yeast cells was assayed as described in (18). Briefly,

173    cells were grown overnight in liquid YPD medium at 30°C with aeration. Then, 2 µL of each

174    culture was spotted onto YPD10 plates (YPD agar supplemented with glucose to a final

175    concentration of 10% w/v), allowed to absorb, and incubated overnight at 30°C. The plates were

176    then covered with 6.5 mL soft-agar (0.5% agar in 300 mM Tris and 187 mM glutamate, pH 8.3).

177    Upon solidification of the soft agar, a second soft-agar overlay was prepared by mixing 3.2 mL

178    1% agar with 3.2 mL of a staining solution composed of 30 mM Tris/18.75 mM glutamate (pH

179    8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL phenazine methosulfate, 7 U

180    glutamate pyruvate transaminase, and 7 U L[+]-lactate dehydrogenase (all LASSO components

181    were from Sigma-Aldrich, St. Louis, MO). Strains producing lactic acid formed purple halos

182    within 10 min, the color of which darkened with increasing incubation time at room temperature.

183    Cells not producing lactic acid never formed halos.

184    *2.5. Multi-well lactic acid production assay*

185         Because only a small number of strains can be tested for lactic acid production on a

186    single plate in the LASSO assay, we also adapted it for use in multi-well plates. Briefly,

187    individual strains were grown overnight in 100 µL YPD10 medium in the wells of 96-well plates

188    at 30°C with aeration in a BioTek Synergy H1 plate reader. To avoid evaporation of the medium,

189    50 µL mineral oil was used to overlay each well. Then, 100 µL of staining solution (30 mM

190    Tris/18.75 mM glutamate (pH 8.3), 2.5 mM NAD, 0.5 mg/mL nitrotetrazolium blue, 125 µg/mL

191    phenazine methosulfate, 1 U/mL glutamate pyruvate transaminase, and 1 U/mL L[+]-lactate

192    dehydrogenase) was added to each well and mixed by agitation in the plate reader. The reaction

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193    proceeded for ≥ 10 min at room temperature, and the presence of lactic acid was indicated by the

194    gold colored solution turning green (and eventually blue with extended incubation).

195    *2.6. Gas chromatography-mass spectrometry (GC-MS) analysis of lactic acid*

196

197

198    **3. Results**

199    *3.1. Establishment of the yeast bank*

200        We previously reported an initial description of our bio-prospecting and characterization

201    of wild yeasts for use in the brewing industry (5). Here, we present the updated culture

202    collection. In all, 284 strains (Supplementary Table 1) from 54 different species in 26 genera

203    (Table 1) were collected, mostly from the American Midwest. Over 68% (195 strains) were from

204    various locations in Indiana alone (Supplementary Table 1). Species known to have brewing

205    potential, *e.g.*, *S. cerevisiae* and *B. bruxellensis*, were isolated from many sources, and the 37 *S.*

206    *cerevisiae* strains recovered made it the most frequently isolated species (Table 1). However,

207    these yeasts represented a minority (~13% *S. cerevisiae* and < 3% *B. bruxellensis*) of the total.

208    Instead, species in the *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Pichia*, *Torulaspora*, and

209    *Wickerhamomyces* genera dominated (Table 1).

210        To determine the relative usefulness of these strains in beer brewing, small laboratory-

211    scale beer fermentations were performed for each isolate. Most strains were able to metabolize

212    maltose (and other wort sugars) into ethanol and carbon dioxide, but the strains greatly varied in

213    their levels of attenuation, ability to flocculate, and in the sensory profiles of the aromatic and

214    flavor compounds that they produced (data not shown). Interestingly, these variations were noted

215    both between species and among isolates of the same species, even when two distinct strains of

216    the same species were isolated from one sample. Notable exceptions to this were all of the *Pichia*

217    species. The sensory profiles of beers fermented with these yeasts were uniformly abhorrent,

218    with the most colloquial but apt descriptor being "demon sulfur fecal juice." Regardless, many of

219    the other yeasts displayed brewing potential that was commensurate with our control *S.*

220    *cerevisiae* ale yeast strain WLP001. Reports of the detailed analysis of the brewing

221    characteristics of exemplary single strains and strain groups are forthcoming. Herein though, we

222    focus on a group of yeasts that appeared to be capable of heterolactic fermentation of sugar into

223    lactic acid, ethanol, and $CO_2$.

224    *3.2. Five heterofermentative yeast species.*

225        During our sensory analyses of the laboratory-scale fermentations above, we noted that

226    many of the strains were producing beers that were characterized as tart or sour (Table 2), akin to

227    styles that are produced with the aid of LAB (19). When we grouped these strains, we found that

228    they were all members of five species: *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*,

229    and *W. anomalus* (Supplementary Table 1 and Fig. 1). To determine if this apparent

230    heterofermentative activity was specific to evolutionarily closely related yeasts, we aligned the

231    sequences of the D1/D2 variable region of their ribosomal DNA and plotted a phylogenetic tree.

232    As shown in Figure 1, three of the species (*H. vineae*, *L. fermentati*, and *L. thermotolerans*) are

233    closely related to ale yeast (WLP001), but the other two (*S. japonicus*, and *W. anomalus*) form

234    more distinct clades.

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235     Regardless of their evolutionary relationships, the strains listed in Table 2 and other

236     isolates of the same species (data not shown) varied in their fermentative activities. Their levels

237     of attenuation varied from 40-83%, with decreases in the initial pH of 5.0 to as low as 3.21.

238     Although some of these differences may be attributable to differences among the species

239     themselves, intra-species differences were also noted, especially during sensory analyses. For

240     instance, *L. thermotolerans* YH73 produced a "very sour" flavor with berry notes, but the same

241     beer fermented with *L. thermotolerans* YH79 was characterized as only "slightly tart" yet clean

242     and rounded (Table 2).

243     *3.3. Characterization of strains WYP39, YH72, YH82, YH140, and YH156*

244     Because the trial fermentations above were performed in a high-throughput manner, we

245     chose representative strains for more in-depth investigation. We compared their colony and

246     cellular morphologies, sought to verify that they were producing lactic acid, and performed

247     larger-scale fermentations. Figure 2A shows representative colony morphologies for the strains

248     grown on YPD and WLN agar. The wild *S. cerevisiae* strain YH166 was included for

249     comparison. On YPD plates, all strains produced colonies that were creamy white, round, and

250     raised. The *L. fermentati* WYP39 and *L. thermotolerans* YH140 colonies were also glossy, while

251     the others had comparatively matte surfaces. Based on colony size, the *H. vineae* YH72, *W.*

252     *anomalus* YH82, and (especially) *S. japonicas* YH156 strains grew slower on YPD medium than

253     the other species. However, all strains grew at similar rates on WLN agar. All of the species also

254     took up the bromocresol green pH indicator dye from the WLN medium, with YH166 and

255     YH140 attaining a pale mint green colony color, YH156 assuming a green shade, and WYP39,

256     YH72, and YH82 colonies turning dark green. The YH72 and YH82 colonies additionally

257     displayed pale margins on WLN plates.

258     We next observed the cellular morphologies of the six species above using differential

259     interference contrast (DIC) microscopy (Fig. 2B-G). As expected, all strains except YH156

260     appeared as typical round-to-ellipsoid budding yeast, though YH72 failed to display the

261     "bowling pin" shape usually associated with apiculate yeast like *H. vineae*. Additionally, the

262     YH72 and YH82 cells displayed the propensity to clump together (Fig. 2C and D), even after

263     dilution and vigorous vortexing. This may be an effect of growth with aeration, as these strains

264     were no more flocculant during fermentation than WYP39 (Table 3) or YH166 (data not shown).

265     The YH156 cells were much larger than the other species analyzed and displayed the usual

266     *Schizosaccharomyces* rod-shaped morphology (Fig. 2F). We also observed evidence of

267     microhyphae (20) among these cells. This mycelial mode of growth may explain the excellent

268     flocculation of YH156 during fermentation (Table 3) (21).

269     *3.4. Strains WYP39, YH72, YH82, YH140, and YH156 produce lactic acid*

270     We further sought to determine if strains WYP39, YH72, YH82, YH140, and YH156

271     were truly producing lactic acid during fermentation, rather than one or more other secondary

272     metabolites that yield a tart/sour flavor (22). Using the LASSO assay for lactic acid production

273     by yeast (18), we found that all five strains produced lactic acid (denoted by dark halos in Fig.

274     3A), similar to the *Lactobacillus plantarum* positive control. In contrast, the *S. cerevisiae*

275     WLP001 negative control failed to generate a halo. Because the LASSO assay can only be used

276     for a limited number of strains on a single plate, we adapted it into a multi-well plate assay (Fig.

277     3B). Here, lactic acid production is evident by the golden-colored assay medium turning green,

278     as indicated by the LAB controls. Again, WLP001 failed to generate detectable lactic acid, as did

279     a common research strain of *E. coli*. However, multiple tested isolates of *L. thermotolerans*, *L.*

280     *fermentati*, *H. vineae*, *S. japonicus*, and *W. anomalus* did test positive for lactic acid production.

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281    Some individual *L. thermotolerans* strains failed to generate lactic acid (wells 2 and 4) or did so

282    slowly, as certain wells were just beginning to turn green (well 3) when the image in Figure 3B

283    was acquired. These results correspond with sensory analysis of beers fermented with the various

284    *L. thermotolerans* strains, which ranged from not sour to very tart (Table 2 and data not shown).

285    *3.4. Analysis of beers fermented with lactic acid-producing yeasts*

286        To monitor the activities of WYP39, YH72, YH82, and YH156 in larger-scale

287    fermentations, we inoculated these strains into glass carboys containing 19 L (5 gal) each of an

288    identical blonde wort. Because the beer brewing capabilities of a *L. thermotolerans* strain were

289    recently described (14), we omitted strain YH140 from these assays. All of the strains had short

290    lag times (*i.e.*, the approximate time from inoculation to the first visible signs of fermentation)

291    ranging from 6-14 h (Table 3). These lag times to fermentation were similar to that of WLP001

292    inoculated in a similar blonde wort (~12 h, data not shown). However, the kinetics of the full

293    fermentation differed for each lactic acid-producing yeast. WYP39 had the shortest lag time and

294    fermented rapidly for 2 weeks, at which point it slowed considerably and required an additional 2

295    weeks to reach a final gravity of 0.099 (Table 3). YH72 was a slow and steady fermenter,

296    attaining a terminal gravity of 1.000 after 3 weeks. YH82 was similar, but required a full 4 weeks

297    to reach a final gravity of 1.001.

298        The YH156 strain displayed the most variant fermentative characteristics. After reaching

299    a vigorous state of fermentation at 14 h (Table 3), popcorn-like clusters of cells formed and

300    floated around within the fermenter (Supplemental Fig. 1). Eventually, they settled into a

301    mountainous pile against one side of the carboy before compacting down into a yeast slurry with

302    a typical appearance on the bottom of the fermenter. The fermentation reached a final gravity of

14

303    0.099 approximately 27 days after inoculation. The final pH of each beer was recorded and

304    varied from a low of 3.20 to a high of 3.74. Sensory analyses of each beer were conducted

305    (Supplemental Fig. 2), and the tasting notes are discussed in Sections 4.1-4.5 below.

306         We also quantified the concentration of L-lactic acid present in each beer by GC-MS

307    (Fig. 3C). We used Cauldron, a commercially available sour beer made by mixed fermentation of

308    yeast and LAB (17), as a positive control for lactic acid production; it contained 100.54 mM

309    lactate. Based on a lactate standard curve, WYP39, YH72, YH82, and YH156 produced 10.02,

310    35.69, 29.05, and 50.09 mM lactate, respectively. These results contrast with those from the

311    LASSO assay in Figure 3A, where YH156 displayed the least evidence of lactic acid production.

312    Because the lactic acid production occurred in the presence of oxygen in the LASSO assay, this

313    may indicate that strain YH156 is Crabtree negative or only weakly Crabtree positive, *i.e.*,

314    requiring anaerobic conditions for fermentation (see (23) and references therein). It should also

315    be noted that the beers analyzed by GC-MS were fermented in a brewery that utilizes LAB, and

316    thus, it remains a formal possibility that they may have been inadvertently contaminated by other

317    organisms that can generate lactic acid. However, the results in Figures 3A and 3B are from pure

318    cultures (as judged by post-fermentation plating, microscopy, and PCR analyses of the yeast

319    slurry; data not shown and Supplemental Fig. 3) of WYP39, YH72, YH82, and YH156. These

320    cultures also still yielded lactic acid during fermentation in the presence of antibiotics or 75 IBU

321    wort in a laboratory setting (data not shown), strongly suggesting that LAB contamination is not

322    the source of the lactic acid.

323    **4. Discussion**

15

324      The next phase in the "local" movement in the beer industry will be the isolation and use

325    of local yeast strains in brewing. Indeed, in the U.S., nearly all commercially available ale and

326    lager strains are of European origin, so no American beer will ever truly be local without the

327    inclusion of New World yeast. Here, we detailed our regional yeast bio-prospecting efforts and

328    the initial characterization of nearly 300 strains for use in fermentation. Within this strain bank,

329    we uncovered five species that generate lactic acid and ethanol during primary fermentation and

330    suggest that they can be used in a novel, LAB-free beer souring method (see Section 4.6. below).

331    *4.1.* H. vineae

332      As the species name suggests, *H. vineae* is typically associated with wine, where it has

333    previously been investigated alone and in combination with *S. cerevisiae* for grape must

334    fermentation (24-26). The strains previously tested are notable for the production of high levels

335    of 2-phenylethyl acetate, which is an aromatic compound that lends floral, fruity, and/or honey-

336    like notes to wine (25). Although yeasts in the *Hansenia* genus are the predominant species

337    found on grapes (27-29), they are also found elsewhere. For instance, *H. vineae* has been isolated

338    from agave plants, must, and cooked agave, as well as throughout tequila distilleries (reviewed in

339    (30)). Here, we enriched for *H. vineae* strains from the bark of oak, sycamore, and ash trees,

340    blueberries, and grapes (Supplementary Table 1).

341      To our knowledge, this is the first report of pure cultures of *H. vineae* being used to

342    ferment beer. Strain YH72 ferments slowly relative to typical commercially available ale yeasts,

343    but reached high levels of attenuation after only two weeks (Table 2) and further attenuated with

344    additional fermentation time (Table 3). The beers produced by short fermentations with YH72

345    were slightly sour but clean and highly drinkable, with notes of apple cider. Longer fermentation

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346    yielded very sour beer with a pH (3.23) and acidic bite reminiscent of beers produced with LAB,

347    as well as stone fruit notes (Table 3, Supplementary Fig. 2). Preliminary experiments also

348    indicate that repeated exposure to wort fermentation conditions may adapt YH72 to attenuate

349    more quickly (M. Bochman, observations). Based on these results, other closely related species,

350    such as *H. guillieromondii*, *H. occidentalis*, *H. opuntiae*, *H. osmophila*, *H. uvarum*, and *H.*

351    *valbyensis*, should be investigated for their use as brewing strains and the production of lactic

352    acid. We isolated several strains from three of these species (Table 1) and plan to characterize

353    their fermentative capabilities.

354    *4.2.* L. fermentati

355       Very little is known about *L. fermentati*, especially with regard to beverage fermentation.

356    In the environment, *L. fermentati* is generally associated with leaves and decaying plant matter

357    (31,32). We isolated our strains from the bark of live trees such as oak, hickory, and birch

358    (Supplementary Table 1). Industrially, this species of yeast has also been found in fermented

359    (wine (33), cachaça (34), and water kefir (35)) and non-fermented beverages (coconut water and

360    fruit juices (36)). However, its effects on the sensory characteristics of these beverages are

361    largely unknown.

362       As with *H. vineae*, this also appears to be the first report of beer fermented with pure

363    cultures of *L. fermentati*. Our laboratory-scale test fermentations indicated that strain WYP39

364    displayed decent wort attenuation for a wild strain (60%, Table 2), and longer fermentation in a

365    larger-scale fermentation yielded a dry product (Table 3). The final pH was modest compared to

366    other sour beers (Table 3), creating a flavor that was more tart than sour, but this was

367    accentuated by light pineapple and mango flavors. There are many species in the *Lachancea*

368  genus (37). Based on the desirable brewing characteristics of *L. fermentati* and *L. thermotolerans*

369  (discussed below in Section 4.3. and in Domizio *et al*. (14)), it will be interesting to assess the

370  activities of these other species during beer fermentation. Thus, our four isolates of *L. kluyveri*

371  (Table 1) will be the focus of future work.

372  *4.3.* L. thermotolerans

373      Like the closely related *L. fermentati*, *L. theromtolerans* also colonizes leaf surfaces (32),

374  as well as insects (38,39) and sugarcane (40). We isolated our strains from the barks of numerous

375  tree species, mulberries, bell pepper, and persimmon fruit (Supplementary Table 1). Various

376  strains of *L. theromtolerans* have been studied for their effects on wine fermentation (reviewed

377  in (28)), though usually in co-fermentations with *S. cerevisiae* (*e.g.*, (41,42). Recently, *L.*

378  *thermotolerans* strain Lt101 was shown to be a viable yeast for beer production (14), and the

379  same group also found that three *L. thermotolerans* strains including Lt101 produce lactic acid

380  during fermentation. Although this is similar to our observations (Table 2 and data not shown),

381  the strains investigated by Domizio *et al*. (14) only decreased the pH of wort from 5.66 to 4.28-

382  3.77 during fermentation. Most of their experiments yielded final pH values in the 4.17-4.3

383  range, however, which is similar to the pH decrease caused by *S. cerevisiae* UCD 915. Our *L.*

384  *thermotolerans* isolates that produced noticeably tart beers reached terminal pH values of ~3.35

385  (Table 2 and data not shown). These discrepancies are likely due to differences in the

386  experimental set ups, as well as strain-to-strain variability, which is discussed in Section 5

387  below.

388  *4.4.* S. japonicus

18

389    *S. japonicus* is closely related to *S. pombe*, which was originally isolated from African

390    millet beer (reviewed in (43)). *S. japonicus* itself was first isolated from strawberries in Japan

391    (44), and we recovered two strains from the bark of an oak tree in northeastern Pennsylvania

392    (Supplementary Table 1). Unlike the other yeast species investigated here, *S. japonicus* is a

393    fission yeast rather than a budding yeast (Fig. 2E). This difference in physiology is borne out of

394    genomic differences, as *S. japonicus* is as evolutionarily distant from *S. cerevisiae* as each yeast

395    is from humans (45).

396    Regardless, *S. japonicus* is associated with indigenous fermented beverages (*e.g.*, kaffir

397    beer, plantain beer, palm wine, sugar cane wine, and sake) around the world (46), wine

398    production (47), and was isolated from spontaneously fermented beer in North Carolina (48).

399    However, no characterization of the beer is available. Thus, this is the first rigorous report of *S.*

400    *japonicas* used for primary fermentation of beer. The attenuation levels of strain YH156 were

401    excellent in both laboratory- and large-scale fermentations (Table 2 and 3), and the aroma and

402    flavor profiles of these beers included common descriptors of sour, fruity, and stone fruit.

403    Individual tasters identified green apple Jolly Rancher, tart apple, pear, pineapple, and peach

404    notes.

405    The *Schizosaccharomyces* genus includes four member species: *S. cryophilus*, *S.*

406    *japonicus*, *S. octosporus*, and *S. pombe*. We isolated two *S. japonicus* and four *S. pombe* strains

407    (Table 1), but only the *S. japonicus* YH156 strain yielded appreciable lactic acid production

408    during our trial fermentations (Tables 2, 3, and data not shown). Regardless, we believe that it is

409    worthwhile to test other strains of these species, as well as *S. cryophilus* and *S. octosporus*, for

410    their use as in the primary fermentation of beer.

411   *4.5.* W. anomalus

412   *W. anomalus*, previously known as *Saccharomyces anomalus* (49), has multiple roles in

413   the biotechnology, agriculture, and food production fields. It is often found in association with

414   grain and is especially useful to inhibit storage molds during malting (50). Concerning beverage

415   fermentation, *W. anomalus* is generally referred to as a beer spoilage organism (51), but it is also

416   necessary for cocoa and coffee bean fermentation (52). We isolated 17 strains of *W. anomalus*

417   from diverse sources: fruit, tree bark, beer, flowers, soil, and spent grain (Table 1 and

418   Supplementary Table 1).

419   Although *W. anomalus* has been investigated for its use in apple wine and hard cider

420   production (53,54), the only work involving beer has focused on the spoilage properties of this

421   species (51). As with *S. japonicus* YH156 above, the YH82 strain of *W. anomalus* yielded

422   excellent attenuation at both fermentation scales tested (Tables 2 and 3). This strain produced a

423   less intense sour character than others, but the beer was characterized as clean, aromatic, and

424   fruity with notes of pear, apple, and apricot. Of all of the lactic acid-producing yeasts described

425   herein, *W. anomalus* is perhaps the most exciting because the *Wickerhamomyces* clade contains

426   over a dozen species, with more being discovered and characterized with regularity (55).

427   Although we did not isolate closely related species to *W. anomalus*, our small collection of 17

428   strains is nonetheless an excellent place to begin to determine the usefulness of these yeasts in

429   beer fermentation.

430   *4.6. Primary souring*

431   There are two general methods by which sour beers are produced: kettle souring and

432   mixed culture fermentation (Fig. 4) (19). Kettle souring is the more rapid and modern technique.

433    During kettle souring, unhopped or lightly hopped wort is produced in a brew kettle as normal,

434    but then it is only partially cooled to approximately 40-45°C. This temperature favors the growth

435    of LAB, which can be introduced by inoculation with pure cultures or the addition of grain. The

436    LAB then sour the wort in the brew kettle to the desired pH, which usually occurs over the

437    course of 24-48 h. The soured wort in the kettle is ultimately boiled a second time to kill the

438    LAB, and hops can be introduced at this point. The wort is then transferred to a fermenter and

439    (typically) inoculated with *S. cerevisiae* for primary fermentation. Although this method affords

440    brewers tight control over acid production (souring can be stopped at any time via boiling) and is

441    less time consuming that mixed culture fermentation (below), it also has inherent weaknesses.

442    First, the entire souring process occurs in the brew kettle, so it prevents additional wort

443    production in that vessel. Indeed, kettle souring is often relegated to weekends when small

444    breweries are otherwise not in production mode. Second, boiling the wort after it has been soured

445    drives off volatile aromatics that may also have been produced during souring, yielding beers

446    that are described and criticized as lacking in depth and character. To combat some of these

447    sensory downsides, some brewers are now barrel aging kettle sours to impart oak complexity to

448    the final beers.

449        Souring by mixed culture fermentation is the more traditional method. In this process

450    (Fig. 4), lightly hopped wort is produced in the brew kettle and transferred to a fermenter. There,

451    it can be inoculated with *S. cerevisiae* for primary fermentation. In some cases, LAB are added

452    prior to or concurrently with *S. cerevisiae* (or *Brettanomyces* spp. in 100% "Brett" beers). If the

453    LAB are added at this stage, souring begins during primary fermentation. After the yeast has

454    attenuated the beer to the desired level, it is then barrel aged for months or years until it attains a

455    low pH and complex flavor profile. Barrel aging is another stage at which LAB and

21

456    *Brettanomyces* spp. can be added (either resident in the barrels or as pure inocula) to induce

457    souring. Mixed culture souring produces more complex and nuanced flavors in beer than kettle

458    souring, but it suffers from a huge time lag from wort production to the final beer, and it requires

459    a large space dedicated to housing the barrels.

460        Here, we propose a third paradigm for sour beer production that we call primary souring.

461    In this method, the wort is inoculated with a yeast capable of heterolactic fermentation rather

462    than *S. cerevisiae*, and souring occurs during primary fermentation in the absence of LAB (Fig.

463    4). The yeast strains described in Tables 2 and 3 and in the text above did not display rapid

464    fermentation kinetics like commercially available ale yeasts, but they still completed

465    fermentation within a month, displaying excellent levels of attenuation and medium-to-high

466    flocculation (Table 3). Further, the sensory profiles of the beers were superior to kettle soured

467    beer, displaying both lactic tartness and fruity aromatic and flavor notes. Compared to the sour

468    production methods above, primary souring is beneficial in that it frees up the brew kettle and

469    does not require lengthy aging in barrels, though oak aging is a possibility after primary

470    fermentation (Fig. 4). Perhaps most alluringly, primary souring does not require the introduction

471    of bacteria into the brewery, and preliminary tests suggest that *H. vineae*, *L. fermentati*, *L.*

472    *thermotolerans*, *S. japonicus*, and *W. anomalus* can be eliminated as easily as *S. cerevisiae* from

473    brewing equipment using standard clean-in-place protocols (data not shown). As is typical of

474    yeasts, these species are also hop tolerant, enabling more liberal use of hops in wort production

475    for sour beers. However, it should be noted that yeast growth can be inhibited by hop iso-$\alpha$-acids

476    in acidic milieus (56), so the absolute levels of hop tolerance will likely vary by strain and the

477    desired pH of the sour beer.

478

479 **5. Conclusions and Outlook**

480    We set out to isolate and characterize new yeasts for use in beer fermentation, to mine the

481    natural treasure trove of wild strains for novel aromas and flavors that have been missed by

482    focusing on the currently used highly genetically related brewing yeast strains of *S. cerevisiae*.

483    Here, we reported the results of our local bio-prospecting efforts and highlighted the discovery of

484    a set of yeast species that produce both lactic acid and ethanol during primary fermentation. It is

485    unclear how widespread this heterolactic fermentation phenotype is among ethanol-tolerant

486    yeasts, but its presence in the fission yeast *S. japonicus* and budding yeasts like *H. vineae*, which

487    are separated by ~1 billion years of evolution (45), may indicate that heterolactic fermentation is

488    an ancient and conserved metabolic process among single-celled fungi. Arguing against this

489    hypothesis is the lack of detectable lactic acid production by related strains of the same species,

490    *e.g.*, differences among *L. thermotolerans* isolates ((14) and data not shown). Perhaps the genes

491    enabling lactic acid production are on an extrachromosomal element only found in certain

492    strains, or they may be epigenetically silenced in some cases but not others. The latter possibility

493    is intriguing given the prion-based heritable changes in sugar metabolism that are caused by

494    bacteria communicating with yeast cells via lactic acid (57,58). Regardless, it is our hope that the

495    strains described above and the primary souring process put forth here will add strength to the

496    already growing sour beer movement in the U.S. and abroad.

497

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660

661    **Tables**

662    **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 |

28

|  | *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 |

663

664

665 **Table 2. Lab-scale fermentation and sensory results for representative heterofermentative**
666 **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 |

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

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

670 [c] The initial pH was 5.0.

671

672 **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 |

673 [a] WYP39 = *Lachancea fermentati*, YH72 = *Hanseniaspora vineae*, YH82 = *Wickerhamomyces*

674 *anomalus*, and YH156 = *Schizosaccharomyces japonicus*.

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

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

677    **Figure Legends**

678

679    **Figure 1. Evolutionary relationships among the wild strains and a commercially available**

680    **ale yeast.** The D1/D2 rDNA sequences of the indicated strains were aligned, and the

681    phylogenetic relationships among them were drawn as a rooted N-J tree using

682    *Schizosaccharomyces pombe* as the outgroup. From top to bottom, the *S. japonicas* strains are

683    highlighted purple, the *W. anomalus* strains are red, the *H. vineae* strains are green, the *L.*

684    *fermentati* strains are dark blue, and the *L. thermotolerans* strains are light blue. *S. cerevisiae*

685    strain WLP001 is not highlighted and occupies the relative midpoint of the phylogenetic tree.

686    **Figure 2. Colony and cell morphology of the indicated yeast strains.** A) *H. vineae* (YH72), *L.*

687    *fermentati* (WYP39), *L. thermotolerans* (YH140), *S. japonicus* (YH156), and *W. anomalus*

688    (YH82) colony morphology on YPD agar (middle) and WLN agar (right). *S. cerevisiae* strain

689    YH166 was included for comparison. (B-G) Light micrographs of *H. vineae*, *L. fermentati*, *L.*

690    *thermotolerans*, *S. japonicus*, and *W. anomalus* (respectively) taken by differential interference

691    contrast imaging. The black arrow in (F) indicates a probable microhyphae. Magnification =

692    1000x.

693    **Figure 3. Lactic acid production.** A) LASSO assay for lactic acid production by the indicated

694    strains. Cells producing lactic acid develop a dark halo. Images of both the top and bottom of a

695    representative LASSO plate are shown. *S. cerevisiae* WLP001 and LAB (*L. plantarum*) were

696    included as negative and positive controls for lactic acid production, respectively. These results

697    are indicative of three independent experiments using the same strains. B) Multi-well plate assay

698    for lactic acid production. Cell growth medium containing lactic acid turns from gold to green

699    when the enzymatic assay is complete. Multiple strains of *L. thermotolerans, L. fermentati, H.*

700    *vineae*, and LAB were tested, as well as *S. japonicus* YH156 (*Sj*), *W. anomalus* YH82 (*Wa*), *S.*

701    *cerevisiae* WLP001, and *Escherichia coli* DH5α (*E. coli*). WLP001 and *E. coli* served as

702    negative controls for lactic acid production by yeast and bacteria, respectively. *L. plantarum* (left

703    LAB well) and *L. brevis* (right LAB well) were used as positive controls for lactic acid

704    production. C) L-lactate quantification. Typical GC-MS spectra of the lactate standard (solid red

705    line) and isolated lactate from beer samples are shown. HV (solid purple line), WA (solid green

706    line), LF (dashed burnt sienna line), and SJ (dashed black line) represent beers fermented with *H.*

707    *vineae* YH72, *W. anomalus* YH82, *L. fermentati* WYP39, and *S. japonicus* YH156, respectively.

708    "Caul" (solid black line) represents the lactate isolated from Cauldron, a positive control for

709    lactate in beer (See Section 2.6 and (17)).

710    **Figure 4. Comparison of kettle souring, wood-aged souring, and primary souring.** See the

711    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
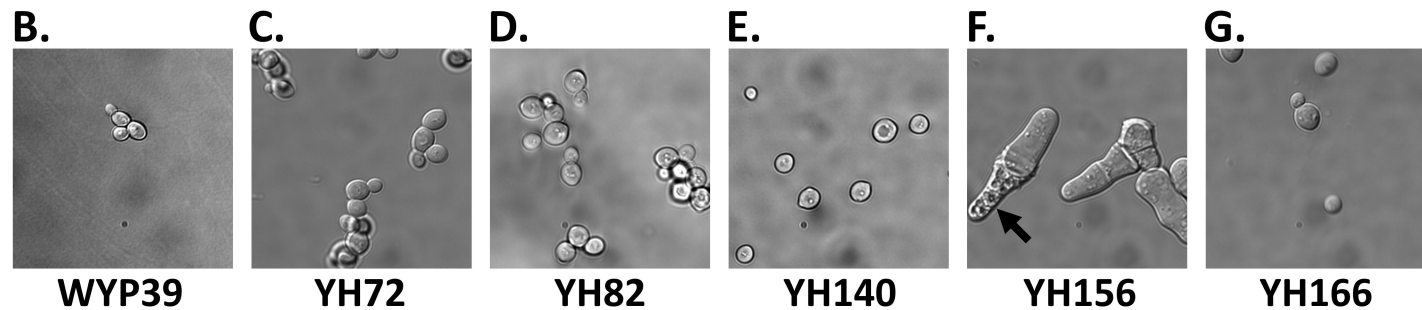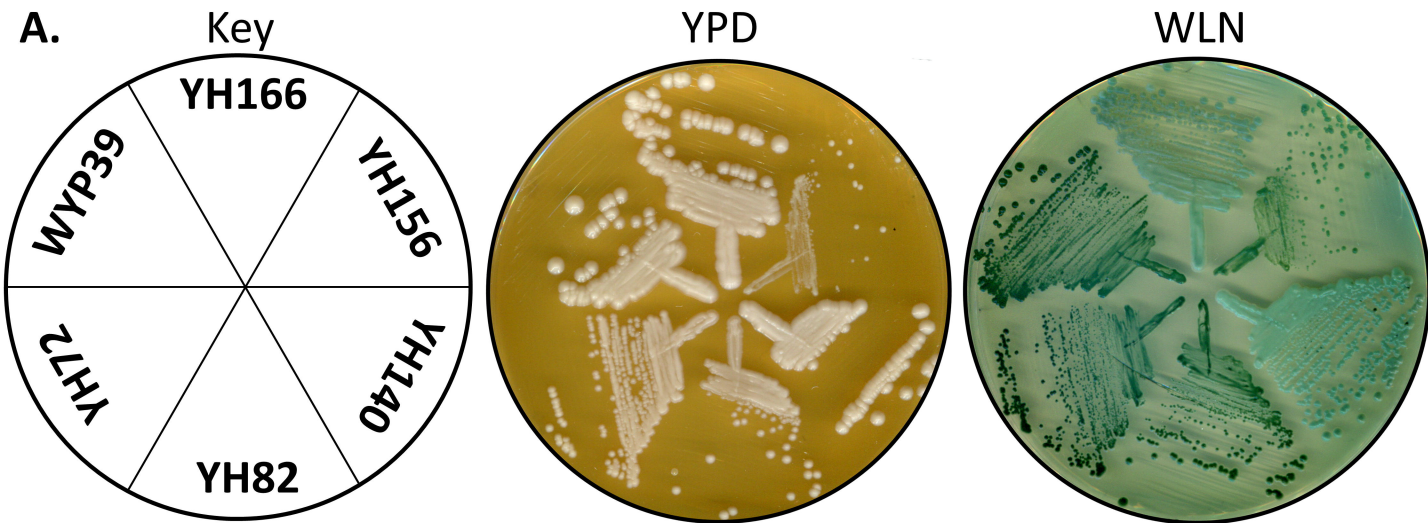YH140
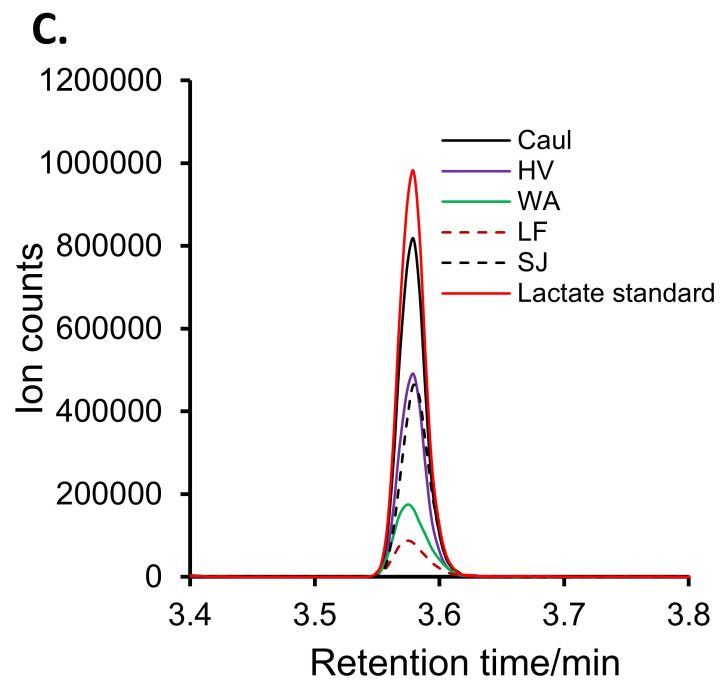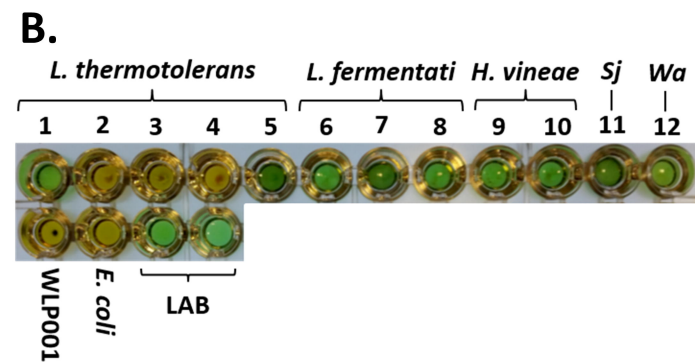YH171
YH121
YH154
YH81
YH128
YH27
YH80
YH26
YH109
YH36
YH39
YH175

**A.** Key | YPD | WLN

| | |
|---|---|
| WYP39 | YH166 |
| YH72 | YH156 |
| YH82 | YH140 |

**B.** WYP39

**C.** YH72

**D.** YH82

**E.** YH140

**F.** YH156

**G.** YH166

**A.**

Key | Bottom | Top

Key:
LAB, WLP001
WYP39, YH72, YH82
YH140, YH156

**B.**

*L. thermotolerans* 1 2 3 4 5 | *L. fermentati* 6 7 8 | *H. vineae* 9 10 | *Sj* 11 | *Wa* 12

WLP001 | *E. coli* | LAB

**C.**

- Caul
- HV
- WA
- LF
- SJ
- Lactate standard

Ion counts (y-axis): 0 to 1200000

Retention time/min (x-axis): 3.4 to 3.8

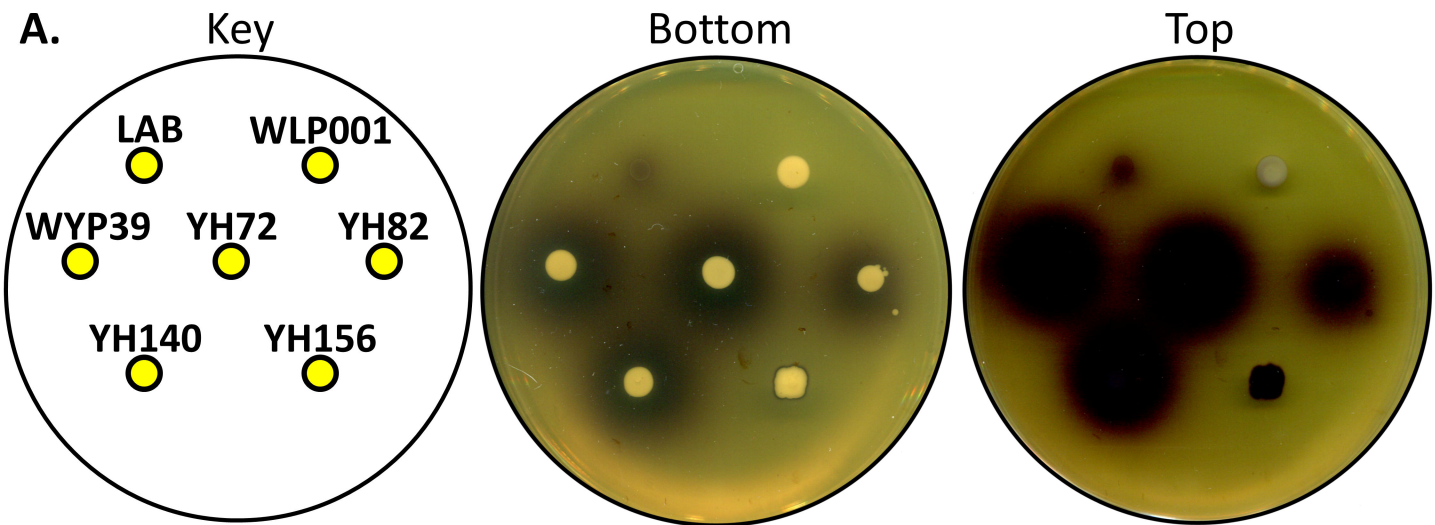| **Kettle souring (fast souring)** | **Mixed culture souring (long-term souring)** | **Primary souring** |
|---|---|---|

**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