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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.

1. Introduction

Currently, we are in the midst of a global craft beer boom, with the number of small independent breweries growing at a tremendous pace (Jones, 2016). This has led to increased competition, not only with the large macrobrewers but among the craft brewers themselves. As such, there is a need in the industry to differentiate oneself from, minimally, other local breweries. This has fueled experimentation with the core beer ingredients of water (Brungard, 2014), malted grain (So, 2014), hops (Bernstein, 2010) and yeast (Osburn et al., 2016), as well as with various adjuncts. Much of this experimentation is also focused on locally sourced ingredients to capture terroir and bolster the local economy (Kallenberger, 2016; Hieronymus, 2016).

Despite this widespread experimentation, the isolation and use of novel yeasts for brewing has lagged behind that of the other ingredients. This is in part due to the easy availability of numerous ale and lager strains from reputable commercial suppliers such as White Labs, Wyeast, and Lallemand (Carpenter, 2014). However,

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

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focusing on two species, *Saccharomyces cerevisiae* for ales and *Saccharomyces pastorianus* for lagers, naturally limits the genotypic and phenotypic variation available in brewing strains. This also translates into a limited palette of aromatic and flavor compounds made by these strains, especially considering their extremely high evolutionary relatedness (Borneman et al., 2016; Gallone et al., 2016).

To overcome this constraint, several laboratories and breweries have begun to culture wild yeasts and characterize their beer fermentation capabilities. Most efforts have focused on wild ale and lager strains (Lee et al., 2011; Sampaio and Goncalves, 2008) to increase the available genetic diversity of strains that naturally display high ethanol tolerance. However, multiple strains of yeasts in the *Brettanomyces*, *Hanseniaspora*, *Lachancea*, and *Pichia* genera (Steensels and Verstrepen, 2014; Domizio et al., 2016; Lentz, 2014) have also been investigated as alternative species for the production of beer.

We also recently began bio-prospecting for wild yeasts with desirable brewing characteristics (Osburn et al., 2016). Here, we report the collection of nearly 300 strains from 26 genera. During trial wort fermentations, we found that strains from five species (*Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans*, *Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*) were capable of heterolactic fermentation of sugar into lactic acid, ethanol, and CO_2. Larger-scale brewing with four strains demonstrated that these yeasts are highly attenuative, flocculate well, yield appreciable levels of lactic acid, and produce pleasant aromatic and flavor compounds. We suggest a new paradigm for sour beer production called “primary souring” that avoids the use of lactic acid bacteria (LAB) and instead relies solely on lactic acid production by a heterofermentative yeast during primary fermentation.

2. Materials and methods

2.1. Strains, media, and other reagents

S. cerevisiae strain WLP001 was purchased from White Labs (San Diego, CA). Wild strains were isolated as described in (Osburn et al., 2016). All yeast strains were routinely grown on yeast extract, peptone, and dextrose (YPD; 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) plates containing 2% (w/v) agar at 30 °C and in YPD liquid culture at 30 °C with aeration unless otherwise noted. All strains were stored as 15% (v/v) glycerol stocks at −80 °C. Media components were from Fisher Scientific (Pittsburgh, PA, USA) and DOT Scientific (Burnton, MI, USA). All other reagents were of the highest grade commercially available.

2.2. Strain identification and phylogenetic analysis

To identify wild yeasts at the species level, frozen stocks were streaked onto YPD plates and incubated at 30 °C until single colonies formed (18–48 h). Colonies were then picked into microcentrifuge tubes containing 100 μL of lysis solution (0.2 M LiOAc and 1% SDS) and incubated in a 65 °C water bath for ≥15 min to lyse the cells. After 300 μL of 100% isopropanol was added to the tubes, they were mixed by vortexing, and the cell debris and genomic DNA (gDNA) were pelleted in a microcentrifuge for 5 min at maximum speed. The supernatant was decanted, and remaining traces were completely removed from the pellets by aspiration. The gDNA was resuspended in 50–100 μL TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA), and a 1-min spin at maximum speed was used to pellet the cell debris to clarify the DNA solution. The variable D1/D2 portion of the eukaryotic 26S rDNA was then amplified by PCR from the gDNA templates using oligos NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) (Lee et al., 2011) and the following cycling conditions: 98 °C for 5 min; 35 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The PCRs were assessed for D1/D2 amplification by running 10% of the reaction volume on 1% (w/v) agarose gels at 100 V (560 bp expected product size). The amplified DNA was then purified using a PCR Purification Kit (Thermo Scientific, Waltham, MA) and quantified using a BioTek Synergy H1 plate reader. The DNA was sequenced by ACGT, Inc. (Wheeling, IL) using primer NL1, and the sequence was used to query the National Center for Biotechnology Information nucleotide database with the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

After species identification, the phylogenetic relationships among the isolated strains of *H. vineae*, *L. fermentati*, *L. thermotolerans*, *S. japonicus*, and *W. anomalus* were determined by aligning their 26S rDNA sequences using ClustalX (Larkin et al., 2007). The alignments were iterated at each step but otherwise utilized default parameters. ClustalX was also used to draw and bootstrap neighbor-joining (N-J) phylogenetic trees using 1000 bootstrap trials; the trees were visualized with TreeView v. 1.6.6 software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The *Schizosaccharomyces pombe* rDNA sequence (GenBank accession HE964968) was included in the alignments as the outgroup, and this was used to root the N-J tree in TreeView. WLP001 was included to determine the relatedness of the wild strains to a commercially available ale yeast.

2.3. Test fermentations

For laboratory-scale fermentations, select yeast strains were streaked for single colonies onto YPD plates as described above and grown to saturation in 4 mL of YPD liquid medium overnight at 30 °C with aeration. The cell count of the starter cultures was approximated by measuring the OD_{660} and converting that value to cells/mL as described at http://www.pangloss.com/seidel/Protocols/ODvsCells.html. In most cases, the saturated overnight cultures reached densities of ~5×10^8 cells/mL. These starter cultures were then used to inoculate ~400 mL of blonde ale wort in 500 mL glass bottles capped with drilled rubber stoppers fitted with standard plastic airlocks. The wort was produced by mashing 65.9% Pilsner (2 Row) Bel and 26.9% white wheat malt at 65 °C (149 °F) for 75 min in the presence of 1 g/bbl $CaCO_3$ and 1.67 g/bbl $CaSO_4$ to yield an original gravity (OG) of 1.044. During the boil, 7.2% glucose was added, as well as Saaz hops to 25 international bittering units (IBUs). The fermentation cultures were incubated at 22.3 ± 0.3 °C (~72 °F) for 2 weeks. Un-inoculated wort was treated as above to control for wort sterility. Prior to bottling into standard 12-oz brown glass bottles, their final gravity (FG) was measured using a MISCO digital refractometer (Solon, OH), and pH was measured using an Accumet AB150 pH meter (Fisher Scientific). Bottle conditioning was conducted as in (Rogers et al., 2016) at room temperature for ≥2 weeks.

Small-batch fermentations were performed at Mainiacal Brewing in Bangor, ME. To produce the test wort, 93.4% two-row base malt and 6.6% carapils were mashed at 66.7 °C (152 °F) to yield an OG of 1.046. During the boil, Loral hops were added to a final concentration of 5.3 IBUs. The wort was then chilled and split into 5-gal portions in separate carboys. Approximately 1×10^{11} cells of the indicated yeast strains were used to inoculate the carboys and allowed to ferment under anaerobic conditions at 21.7 °C (71 °F) for 1 month. Gravity measurements were taken both with a hydrometer and refractometer by standard methods. The final pH was recorded prior to bottling and bottle conditioning as above.