Saccharomyces cerevisiae strain YH166: a novel wild yeast for the production of tropical fruit sensory attributes in fermented beverages

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Abstract

All ales are fermented by various strains of *Saccharomyces cerevisiae.* However, recent whole-genome sequencing has revealed that most commercially available ale yeasts are highly related and represent a small fraction of the genetic diversity found among wild *S. cerevisiae* isolates. This lack of diversity limits the phenotypic variations between these strains, which translates into a limited number of sensory compounds created during fermentation. Here, we characterized a collection of wild *S. cerevisiae*, *S. kudriavzevii*, and *S. paradoxus* strains for their ability to ferment wort into beer. Although many isolates performed well, *S. cerevisiae* strain YH166 was the most promising, displaying excellent fermentation kinetics and attenuation, as well as a tropical fruit sensory profile. Use of this strain in multiple styles of beer suggested that it is broadly applicable in the brewing industry. Thus, YH166 is a novel ale strain that can be used to lend fruity esters to beer and should pair well with citrusy hops in hop-forward ales.

Keywords: *Saccharomyces cerevisiae*; yeast; fermentation; beer

Chemical compounds studied in this article: Ethanol (PubChem CID: 702); glucose (PubChem CID: 5793); maltose (PubChem CID: 6255); sucrose (PubChem CID: 5988); xylose (PubChem CID: 135191)

Abbreviations: EtOH, ethanol; YPD, yeast extract, peptone, and dextrose; NJ, neighbor-joining; OD_{660}, optical density at 660 nm; OG, original gravity; FG, final gravity; ABV, alcohol by volume; NE-IPA, New England-style India pale ale

1. Introduction

Many fermented beverages, including beer, wine, kombucha, and kefir, are produced (at least in part) by the metabolic action of yeasts in the genus *Saccharomyces* (1). These organisms are ubiquitous in such applications due to their naturally high levels of tolerance to ethanol (EtOH), low pH, osmotic stress, and anaerobic conditions (2-4). Of the eight yeasts in the *Saccharomyces* species complex (*S. arboricola*, *S. cerevisiae*, *S. eubayanus*, *S. jurei*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, and *S. uvarum*) (5), *S. cerevisiae* is most commonly found in traditionally fermented beverages and is used industrially for beverage fermentation and bioethanol production (1,6). Indeed, *S. cerevisiae* is used for ale and wine production worldwide.

However, recent research has shown that the *S. cerevisiae* strains currently used for these processes lack in genetic variability. Two different groups performed whole genome sequencing of 212 (7) and 157 (8) *S. cerevisiae* strains, most of which are used to make beer and wine. Both groups found that these industrial strains are extremely genetically similar and display high levels of inbreeding. As such, they represent only a small fraction of the natural genetic diversity found among wild strains of *S. cerevisiae*. This narrow genotypic and phenotypic variation among commercially available strains likely limits the spectrum of sensory compounds produced by these yeasts during fermentation.

In beers that are not heavily hopped, the yeast can account for ≥ 50% of the sensory profile of the finished beverage (9). Thus, wild *S. cerevisiae* strains represent an untapped reservoir of aromas and flavors in beverage fermentation. Here, we characterized a collection of wild *Saccharomyces* strains (10,11) for their evolutionary relatedness to each other and commercially available ale strains, their ability to metabolize mono- and disaccharides, their EtOH tolerance, and ability to ferment wort into palatable beer. Of the isolates tested, *S.*

cerevisiae strain YH166 stood out for its excellent fermentation kinetics, EtOH and osmotic stress tolerance, and pleasing sensory attributes that were reminiscent of tropical fruit. We suggest that the use of wild strains such as YH166 for beverage fermentation will represent the next trend in the ongoing global craft beverage revolution.

2. Materials and methods

2.1. Strains, media, and other reagents

S. cerevisiae strains WLP001 and WLP300 were purchased from White Labs (San Diego, CA). Wild strains were isolated as described in (10). All yeast strains were 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. Phylogenetic analysis

The wild *Saccharomyces* strains were identified at the species level by sequencing the variable D1/D2 portion of the eukaryotic 26S rDNA as described (11). After species identification, the phylogenetic relationships among the strains were determined by aligning their rDNA sequences using ClustalX (12). 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 and WLP300 were included to determine the relatedness of the wild strains to a commercially available ale yeasts.

2.3. Sugar metabolism

The yeast strains were grown by inoculating 5 mL YPD liquid medium with single colonies from YPD plates and incubation overnight at 30°C with aeration. The optical density at 660 nm (OD_{660}) of each culture was determined using a Beckman Coulter DU730 UV/Vis Spectrophotometer. Then, the cells were diluted to an OD_{660} = 0.1 in 200 µL YPD medium containing 2% (w/v) glucose, maltose, sucrose, or xylose in round-bottom 96-well plates, overlaid with 50 µL mineral oil to prevent evaporation, and incubated at 30°C with shaking in a BioTek Synergy H1 plate reader. The OD_{660} of every well was measured and recorded every 15 min for 14-15 h, and these values were plotted *vs.* time to generate growth curves. All growth experiments were repeated ≥ 3 times, and the plotted points represent the average OD_{660} values. Error bars representing standard deviations were omitted for clarity.

2.4. EtOH and glucose tolerance

Ethanol tolerance was measured as above but in 96-well plates containing YPD liquid medium or YPD liquid medium supplemented with 5, 10, or 15% EtOH. Glucose tolerance was likewise assessed in 96-well plates containing YPD liquid medium (2% (w/v) glucose) or YP liquid medium supplemented with 10, 20, or 30% (w/v) glucose.

2.5. Test fermentations

Laboratory-scale fermentations were performed as described (11). Briefly, the yeast strains were grown to saturation in 4 mL of YPD liquid medium and used to inoculate ~400 mL

of blonde ale wort (1.050 original gravity (OG)) in 500-mL glass fermenters fitted with standard plastic airlocks. The fermenting cultures were incubated at ~22°C 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). Bottle conditioning was conducted as in (13) at room temperature for ≥2 weeks. The comparisons between WLP001 and YH166 fermentations were conducted in 1-L glass cylinders (30 cm tall, 7.5 cm inner diameter) for 6-7 days at an average temperature of 23.6 ± 0.3°C. The gravity and alcohol by volume (ABV) were monitored in real time using BeerBug digital hydrometers (Sensor Share, Richmond, VA). Small-batch (15-20 L) fermentations were performed by the Bloomington Hop Jockeys (http://hopjockeys.org) home brewing club (Bloomington, IN). Multiple worts were produced for these trials, which are detailed in the Supplementary Materials.

3. Results

3.1. Phylogeny of the wild Saccharomyces *strains*

As we previously reported (10,11), we have isolated hundreds of wild yeasts with the potential for use in the beverage fermentation industry. Among the strains isolated, many *Saccharomyces* species were uncovered, including 37 *S. cerevisiae*, eight *S. paradoxus*, and one *S. kudriavzevii* (11). Because some of these samples came from locations in and around production breweries, we screened them for potential contamination by commercial strains of brewer's yeast. To do so, we aligned the D1/D2 region of their rDNA and constructed a phylogenetic tree to compare the evolutionary relatedness of the isolated strains with commercial

controls (Fig. 1). We found that the yeasts clustered into six distinct clades (I-VI), with the *S. paradoxus* and *S. kudriavzevii* strains all contained within Clade IV. The two commercial strains WLP001 and WLP300 were grouped into Clade V and appear to be closely related to the YH196 and WYP75 isolates, respectively. This suggests that YH196 and WYP75 could be commercial contaminants, and thus, they were excluded from further analyses.

3.2. Characterization of sugar metabolism, ethanol tolerance, and flocculation.

To begin to triage the isolated *Saccharomyces* strains for those most likely to perform well in beer fermentation, we sought to characterize their abilities to metabolize various sugars, their ethanol tolerance, and how well they flocculate. First, to assay for sugar metabolism, we followed the growth of each strain in rich medium (YP) supplemented with 2% (w/v) of two common monosaccharides (glucose and xylose) and disaccharides (maltose and sucrose). We found that the wild isolates could be phenotypically categorized into four groups, representatives of which are shown in Figure 2A-D. Yeasts in Group 1 could equivalently utilize the preferred sugars glucose and sucrose but displayed only minimal growth in the presence of xylose and maltose (Fig. 2A). Strains in Group 2 were likewise able to metabolize glucose and sucrose, as well as displayed an intermediate level of growth in medium containing maltose (Fig. 2B). The isolates in Group 3 displayed similar growth kinetics and cell densities in the presence of glucose, maltose, and sucrose but only moderate growth in xylose-containing medium (Fig. 2C). Finally, the yeasts in Group 4 grew well in the presence of all four tested carbon sources but achieved the highest cell densities in medium containing glucose or sucrose (Fig. 2D).

Next, ethanol tolerance was similarly assessed by growing strains in YPD medium containing 0-15% ABV. Again, the various strains could be grouped based on their growth curves. As shown in Figure 3A, some strains were insensitive to increasing EtOH concentrations,

158 growing as rapidly and to nearly as great a density in the presence of 15% ABV as in the
159 complete absence of EtOH. Other strains displayed similar sensitivities to all concentrations of
160 EtOH tested, though growth was still evident (Fig. 3B). However, most strains displayed a
161 concentration-dependent sensitivity to EtOH, with higher ABVs increasingly inhibiting growth
162 (Fig. 3C). Regardless, all strains grew to some extent in the presence of 15% ABV (Fig. 3 and
163 data not shown), corresponding to the well-documented natural EtOH tolerance of
164 *Saccharomyces* species (2-4).

165 Flocculation was qualitatively assessed by comparing the rate of cell sedimentation by
166 the wild strains to two commercial controls WLP001 and WLP300 (medium and low
167 flocculation, respectively, see www.whitelabs.com) in small stationary liquid cultures and in
168 small fermenters. In both cases, all of the wild strains displayed medium or higher levels of
169 flocculation (Table 1, Supplementary Table 1, and data not shown). However, we did also note
170 that some of the strains formed rather loose slurries that were easily disrupted, sending cells back
171 into suspension with only gentle agitation.

172 *3.3. Small-scale fermentations*

173 Aside from the strains that metabolized maltose poorly (*e.g.*, YH37; Fig. 2A), the other
174 wild isolates all displayed good beer fermentation potential based on our initial tests. To begin to
175 characterize the brewing capacity of these strains, we performed small wort fermentations with
176 each. We utilized WLP001 was a positive control for levels of attenuation and flocculation, as
177 well as a baseline for our sensory analyses. After two rounds of test brewing and analysis, we
178 chose the most promising strains for additional trials. The full data set can be found in
179 Supplementary Table 1, and representative strains are shown in Table 1.

We found that the *S. paradoxus* isolates ranged in their ability to attenuate from 20-55% (Table 1 and Supplementary Table 1) with an average attenuation across all strains of ~37%. Aside from under-attenuation, the beers produced by *S. paradoxus* all smelled and tasted heavily of adhesive bandages, which was likely due to the production of chlorophenol (14). Thankfully, only two *S. cerevisiae* strains (WYP15 and WYP16) shared this sensory phenotype (Supplementary Table 1). Overall, the *S. cerevisiae* strains displayed better attenuation (average of 69%), though they varied widely from 17-95%. Many of the beers produced were neutral in aroma and flavor, though some were fruity, had a Belgian strain phenolic character, and/or were slightly tart and reminiscent of saison or farmhouse ales. The single isolate of *S. kudriavzevii* attenuated well (68%) and yielded neutral sensory characteristics (Table 1).

Of all of the wild *Saccharomyces* strains that we tested, YH166 repeatedly displayed good brewing characteristics, with excellent attenuation (70-80%), flocculation, and aroma/flavor production (Table 1). In every tasting panel that we conducted, the sensory profiles of the beers made by YH166 were consistently characterized as “tropical”, with notes of guava and green apple. Other strains also displayed similar attenuation and flocculation, but the beers they produced were generally neutral in sensory and comparatively bland when sampled alongside beer fermented by YH166. Thus, we focused on YH166 for further characterization.

3.4. Brewing with YH166

S. cerevisiae YH166 was isolated from a spontaneous fermentation conducted in a vacant lot during the summer of 2015 in Indianapolis, IN (11). This wild fermented beer contained six distinct yeast strains: three isolates of *Brettanomyces bruxellensis* and one strain each of *Candida zeylanoides*, *S. cerevisiae* (YH166), and *Wickerhamomyces anomalus*. YH166 was the fastest growing and most vigorous fermenting strain of the six under laboratory conditions (data not

shown). Indeed, when compared to WLP001 in laboratory-scale fermentations, YH166 reliably reached terminal attenuation >24 h faster, though its terminal ABV (~5.5%) was always slightly less than that produced by WLP001 (~6%; Fig. 4A).

We typically use low gravity wort for laboratory-scale fermentations. However, ale strains are commonly utilized in a variety of beer styles, some of which have very high OGs, such as Russian imperial stouts (15). To determine if YH166 could tolerate high gravity wort, we assessed the growth of this strain in rich medium containing 2-30% (w/v) glucose. As shown in Figure 4B, the lag time to exponential growth increased from < 1 h in 2% glucose to > 5 h in 30% glucose. However, YH166 was able to overcome the osmotic stress of the glucose at all concentrations and grow to high density, suggesting that it is suitable for fermenting worts with a wide range of OGs.

Finally, we assessed the activity of YH166 in a variety of worts and fermentation conditions (see Supplementary Materials) with the help of the Bloomington Hop Jockeys, a local home brewing club. It should be noted that each fermentation experiment was only performed a single time, but we feel that the range of conditions tested is still worthy of report. Consistent with our laboratory-scale fermentations, YH166 performed well in all of these trials and produced aromatic (Fig. 4C) and flavor profiles (Fig. 4D) that were reminiscent of apple/pear and tropical fruit. Contrary to the laboratory-scale experiments, however, these beers were uniformly cloudy or hazy in appearance (data not shown).

4. Discussion

The natural tolerance displayed by *Saccharomyces* species to fermentation stresses such as ethanol, low pH, and anaerobic growth (2-4) have enabled these organisms to dominate most industries that rely on fermentation worldwide. However, the yeast strains that are currently used in these processes are highly genetically related (7,8). We sought to characterize wild *Saccharomyces* strains for their ability to ferment wort into beer to determine if novel sensory characteristics can be found in the untapped array of yeast isolates present in nature.

Based on phylogenetics (Fig. 1) and phenotypic analyses (Fig. 2-3 and Table 1), the strains in our collection of wild yeasts could be divided into a variety of groups. It was our hope that one or more of these groupings would be indicative of isolates with positive fermentation attributes to help direct future yeast hunting efforts. This largely proved not to be the case though. For instance, phylogenetic clade IV was dominated by *S. paradoxus* strains that fermented poorly and/or produced unpalatable beer (Fig. 1), but clade IV also contained *S. cerevisiae* YH193 and *S. kudriavzevii* WYP76, both of which produced quaffable ales. The only strain grouping that was relevant for beer fermentation was Group 1 in sugar metabolism (Fig. 2A). Yeasts in Group 1 utilized maltose poorly and consequently attenuated poorly during fermentation (Table 1 and data not shown). Such isolates will be avoided during our ongoing yeast bio-prospecting by only selecting for strains that can rapidly metabolize maltose.

Our current results also suggest that *S. paradoxus* strains should be avoided for beer fermentation. All eight tested here created a repulsive aroma and taste that was reminiscent of adhesive bandages (Table 1). This is a common off-flavor in beer production that is attributable to chlorinated phenols (16). Very little has been reported in the scientific literature about brewing with *S. paradoxus*, and it has been suggested that this is one of the only *Saccharomyces* species not used commercially for fermentation (17). Perhaps this dearth of information is due to off-

putting sensory profiles produced by *S. paradoxus* strains. A brief survey of online resources indicated that home brewers and craft brewers have successfully used *S. paradoxus* in brewing without encountering an antiseptic or medicinal sensory profile, but these reports cannot be verified. Regardless, we collected all of our *S. paradoxus* strains from the bark of oak trees (11), so chlorophenol production appears to be a common characteristic of wild *S. paradoxus* isolated from this natural reservoir.

Unlike the *S. paradoxus* strains, most of the remaining *Saccharomyces* isolates tested produced beers with neutral or more flavorful and pleasing sensory profiles (Table 1). Not all of them attenuated to high levels, but flocculation matched or exceeded the WLP001 control. Serial re-inoculation of low-attenuating strains into wort for fermentation may help to "domesticate" such strains by adapting them to beer production (18), and ongoing experiments are investigating this issue. Many strains, such as YH166, were well suited to fermentation with no manipulation other than the process of enrichment and pure culturing (10).

We chose to focus on strain YH166 due to its excellent fermentation kinetics and tropical fruit sensory profile. In our laboratory-scale trials, it performed as well as the WLP001 ale control strain (Fig. 4A) and demonstrated excellent resistance to osmotic stress (Fig. 4B), suggesting that it can be used to ferment beers with high OGs. YH166 was also amenable to a variety of beer styles when used by home brewers (see Supplementary Materials) and consistently produced sensory profiles with apple/pear and tropical fruit notes. Interestingly, the home brew experiments uniformly yielded beers that were hazy or cloudy in appearance, in contrast to the high flocculation we found in the laboratory (Table 1). Many factors affect flocculation (reviewed in (19)), and thus additional experiments should be performed to determine the effects of variables such as pH, wort gravity, temperature, and cations on YH166

flocculation. Regardless, this lack of flocculation coupled with otherwise desirable brewing characteristics and fruity sensory attributes suggests that YH166 may be an attractive strain for New England-style India pale ale (NE-IPA) brewing. Indeed, NE-IPAs are cloudy-to-opaque and generally described as juicy and fruity (20). Thus, novel wild brewing strains such as YH166 can make an immediate impact on current trending styles of beer and could lead to the development of new beer styles based around the yeast as the core ingredient.

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335 **Tables**

336 **Table 1. Laboratory-scale fermentation results of select strains.**

Strain	Species[a]	Attenuation[b]	Flocculation	Sensory
YH35	*S. paradoxus*	27%	High	Chlorophenol/bandage
YH37	*S. paradoxus*	44%	Medium	Chlorophenol/bandage
YH38	*S. paradoxus*	41%	Medium	Chlorophenol/bandage, vegetal
YH44	*S. cerevisiae*	44%	Medium	Clean, neutral
YH76	*S. paradoxus*	41%	Medium	Chlorophenol/bandage
YH124	*S. cerevisiae*	31%	Medium	Neutral
YH166	*S. cerevisiae*	70-80%	High	Green apple, guava, dry, slightly tart, tropical fruit
WYP2	*S. cerevisiae*	65-80%	Medium	Clean, neutral, reminiscent of lager
WYP4	*S. cerevisiae*	61%	Medium	Silky mouthfeel, cereal grain notes
WYP5	*S. cerevisiae*	61%	Medium	Saison-like
WYP6	*S. cerevisiae*	75-85%	Medium	Thin, farmhouse-like, pithy, earthy
WYP7	*S. cerevisiae*	70-80%	Medium	Crisp, dry, slightly bitter, reminiscent of champagne
WYP43	*S. cerevisiae*	60-80%	Medium	Clean, fruity esters, Belgian phenolic
WYP76	*S. kudriavzevii*	68%	Medium	Neutral
WLP001	*S. cerevisiae*	70-80%	Medium	Clean, neutral, slightly fruity

337

338 [a] All species are in the *Saccharomyces* genus.

339 [b] Strains displaying a range of attenuation were brewed with up to six times to delineate the
340 range. The others were brewed with at least two times, and the greatest attenuation is reported.

341

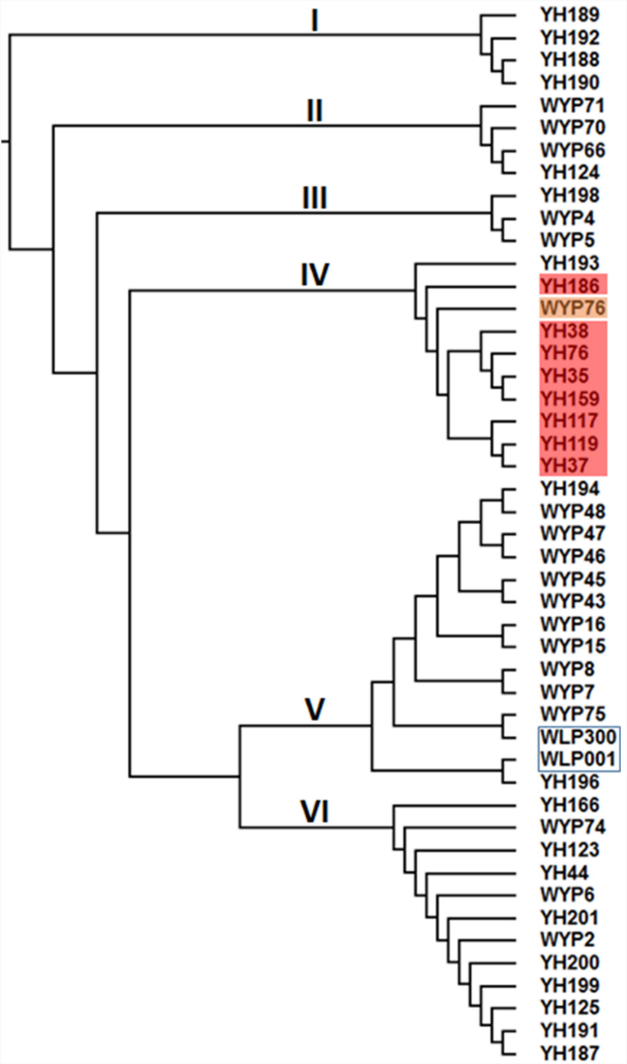
Figure legends

Figure 1. Evolutionary relationships among the wild *Saccharomyces* strains and two commercially available ale yeasts. The D1/D2 rDNA sequences of the indicated strains were aligned, and the phylogenetic relationships among them were drawn as a rooted N-J tree using *Schizosaccharomyces pombe* as the outgroup. Six distinct clades of strains are marked with Roman numerals. The *S. paradoxus* strains are highlighted red, and the *S. kudriavzevii* strain is highlighted orange. The WLP001 and WLP300 commercial strains are boxed in blue.

Figure 2. Growth curves of representative strains utilizing various sugars. Small liquid cultures of the strains indicated on the y-axes were grown in 96-well plates in YP medium supplemented with 2% (w/v) of the indicated sugars. The OD_{660} of each well was monitored with a plate reader. Four different phenotypes were found: A) yeasts in Group 1 grew poorly in the presence of xylose and maltose; B) yeasts in Group 2 displayed a moderate level of growth in the presence of maltose; C) yeasts in Group 3 grew very well in the presence of maltose; and D) yeasts in Group 4 grew well in the presence of all tested sugars. The plotted points in each curve represent the average normalized OD_{660} values of ≥ 3 independent experiments.

Figure 3. EtOH tolerance of representative strains. Small liquid cultures of the strains indicated on the y-axes were grown in 96-well plates in YPD medium or YPD medium supplemented with the indicated amount of EtOH. The OD_{660} of each well was monitored with a plate reader. Four different phenotypes were found: A) tolerance of EtOH up to 15% (v/v), B) similar sensitivities to 5-15% EtOH, and C) concentration-dependent sensitivity to EtOH. The plotted points in each curve represent the average normalized OD_{660} values of ≥ 3 independent experiments.

Figure 4. YH166 displays rapid fermentation kinetics and tolerance to osmotic stress. A) *S. cerevisiae* strains YH166 and WLP001 were independently inoculated into fermenters containing a 1.050 OG wort, and the fermentation kinetics were followed in real time using Wi-Fi-enabled digital hydrometers. SG is plotted on the left y-axis and ABV (%) on the right. The data shown are representative of three independent fermentations for each strain. B) Small liquid cultures of YH166 were grown in 96-well plates in YP medium supplemented with the indicated concentrations of glucose. The OD_{660} of each well was monitored with a plate reader. The plotted points in each curve represent the average normalized OD_{660} values of ≥ 3 independent experiments. C) Spider plot of aroma descriptors for beers fermented with YH166 (see Supplementary Materials for details). D) Spider plot of flavor descriptors for beers fermented with YH166 (see Supplementary Materials for details).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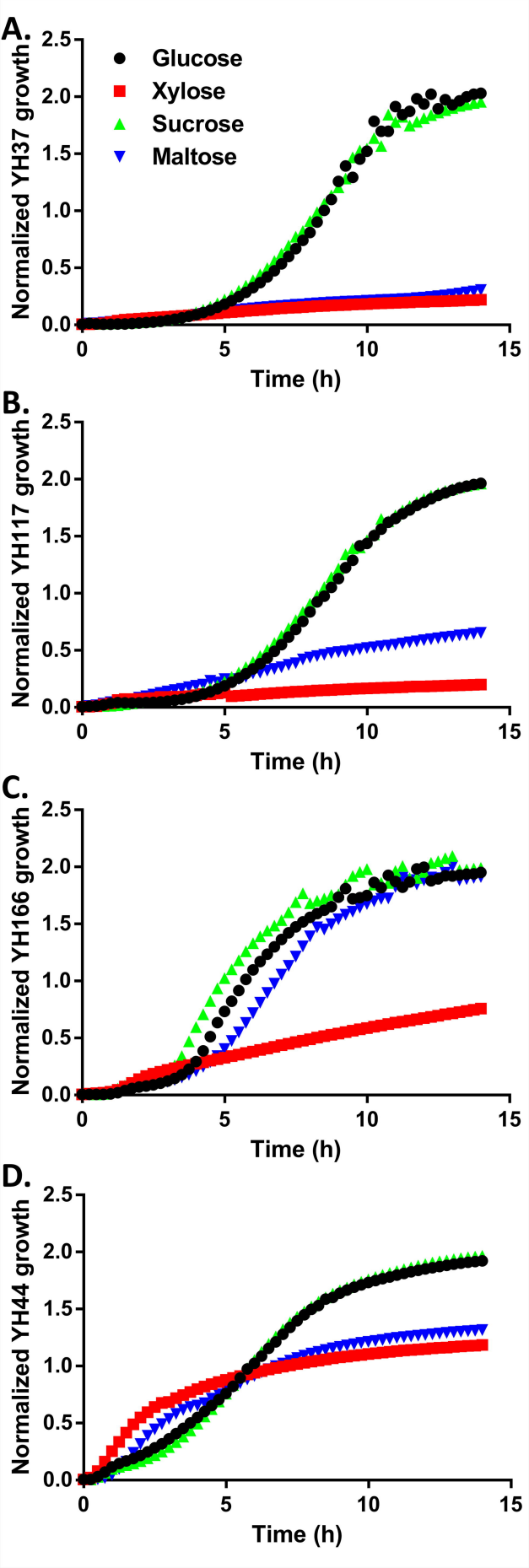
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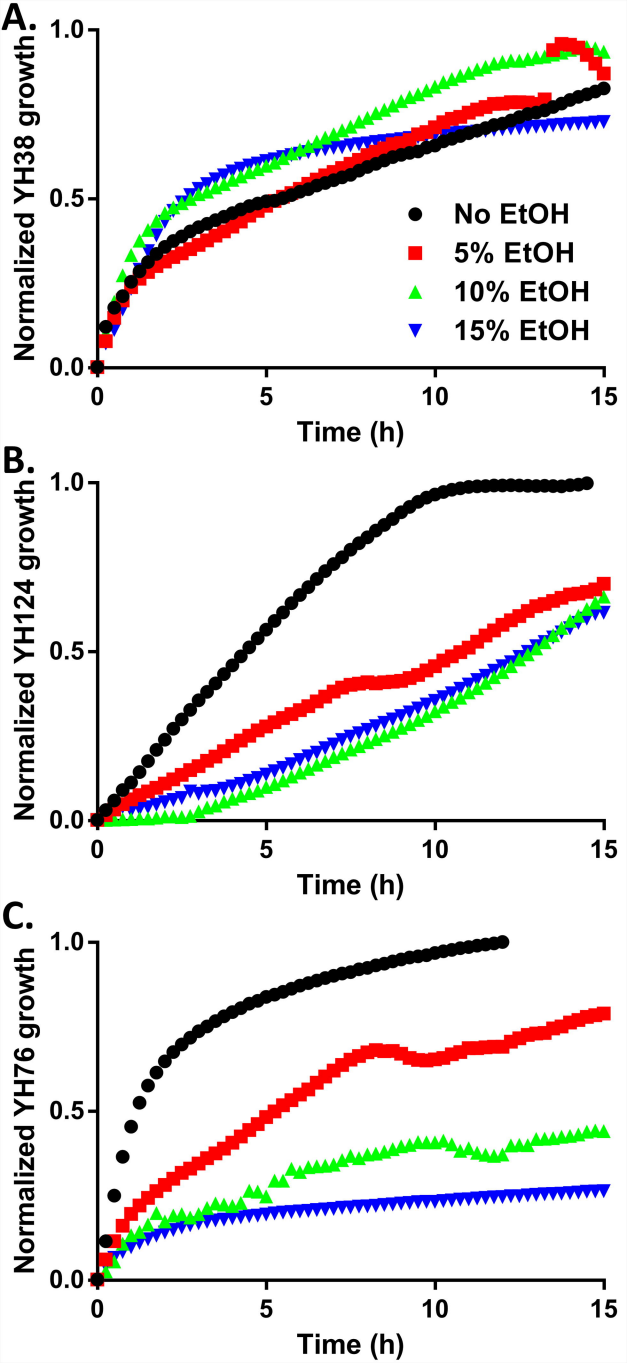
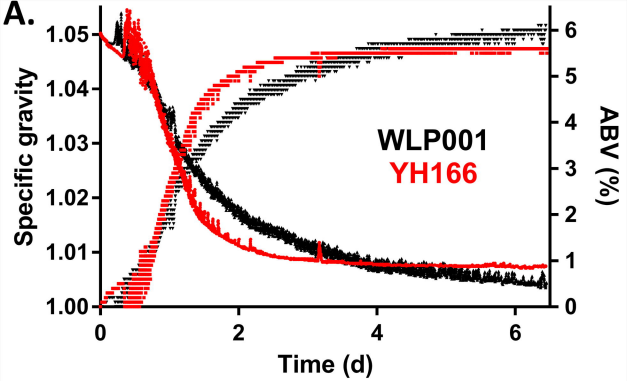
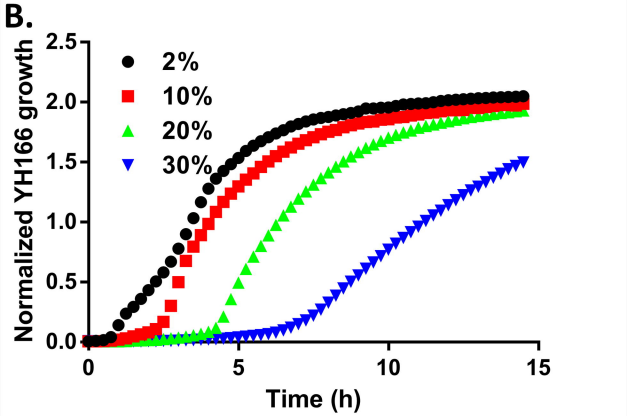
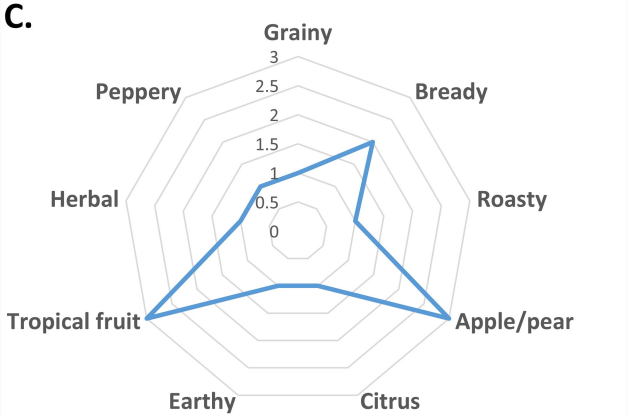
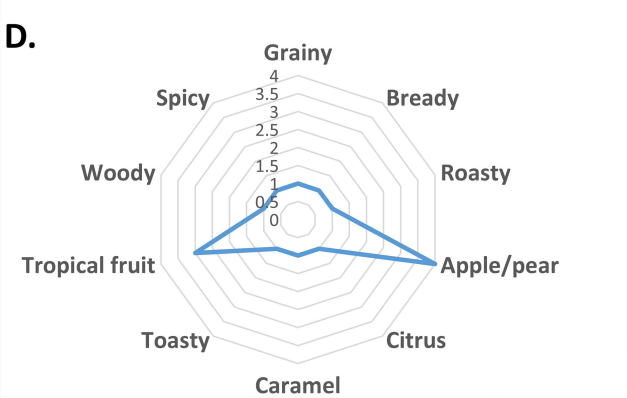
Time (h)
B.
Normalized YH117 growth
Time (h)
C.
Normalized YH166 growth
Time (h)
D.
Normalized YH44 growth
Time (h)


A.
Normalized YH38 growth
1.0
0.5
0.0
No EtOH
5% EtOH
10% EtOH
15% EtOH
0
5
10
15
Time (h)
B.
Normalized YH124 growth
1.0
0.5
0.0
0
5
10
15
Time (h)
C.
Normalized YH76 growth
1.0
0.5
0.0
0
5
10
15
Time (h)


A.
Specific gravity
ABV (%)
WLP001
YH166
Time (d)


B.
Normalized YH166 growth
2%
10%
20%
30%
Time (h)


C.
Grainy
Bready
Roasty
Apple/pear
Citrus
Earthy
Tropical fruit
Herbal
Peppery


D.
Grainy
Bready
Roasty
Apple/pear
Citrus
Caramel
Toasty
Tropical fruit
Woody
Spicy