Food Microbiology 57 (2016) 151–158

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Terminal acidic shock inhibits sour beer bottle conditioning by *Saccharomyces cerevisiae*

Cody M. Rogers [a, 1], Devon Veatch [a, 1], Adam Covey [b], Caleb Staton [b], Matthew L. Bochman [a, *]

[a] Molecular and Cellular Biochemistry Department, 212 South Hawthorne Drive, Simon Hall MSB1, Room 405B, Indiana University, Bloomington, IN 47405, USA
[b] Upland Brewing Company, Bloomington, IN 47405, USA

ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form
29 January 2016
Accepted 27 February 2016
Available online 3 March 2016

Chemical compounds studied in this article:
Lactic acid (PubChem CID: 612)
Ethanol (PubChem CID: 702)

Keywords:
Saccharomyces cerevisiae
Lactic acid
Acetic acid
Sour beer
Bottle conditioning
Acid shock

ABSTRACT

During beer fermentation, the brewer's yeast *Saccharomyces cerevisiae* experiences a variety of shifting growth conditions, culminating in a low-oxygen, low-nutrient, high-ethanol, acidic environment. In beers that are bottle conditioned (*i.e.*, carbonated in the bottle by supplying yeast with a small amount of sugar to metabolize into CO_2), the *S. cerevisiae* cells must overcome these stressors to perform the ultimate act in beer production. However, medium shock caused by any of these variables can slow, stall, or even kill the yeast, resulting in production delays and economic losses. Here, we describe a medium shock caused by high lactic acid levels in an American sour beer, which we refer to as "terminal acidic shock". Yeast exposed to this shock failed to bottle condition the beer, though they remained viable. The effects of low pH/high [lactic acid] conditions on the growth of six different brewing strains of *S. cerevisiae* were characterized, and we developed a method to adapt the yeast to growth in acidic beer, enabling proper bottle conditioning. Our findings will aid in the production of sour-style beers, a trending category in the American craft beer scene.

1. Introduction

Sour beers, traditionally including lambics, *oud bruins*, Flander's red ales, goses, Berliner *weisse*, and more recently American wild ales, represent one of the oldest commercial brewing styles (De Keermaecker, 1996; Tonsmeire, 2014). Historically, such beers relied on spontaneous fermentation by local microflora to metabolize wort sugars into ethanol (EtOH) in a process that can last for several years before bottling. Although most commercially available ales are solely fermented by the yeast *Saccharomyces cerevisiae*, the wild microbes that inoculate sour beers include many species of yeast (*e.g.*, *Saccharomyces*, *Brettanomyces*, and *Hanseniaspora* spp.), as well as lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Spitaels et al., 2014; Tonsmeire, 2014). The metabolic byproducts of these latter microbes acidify the beer, resulting in its characteristic sour flavor (Li and Liu, 2015).

The physiological responses of *S. cerevisiae* to stresses are as varied as the types of stress themselves (*e.g.*, oxidative, osmotic, and EtOH stress) (reviewed in (Gibson et al., 2007; Ingledew, 2009)). However, the general stress response is characterized by the transient upregulation of the expression of ~200 genes that encode proteins such as molecular chaperones, which enable the yeast to deal with changes in their environment (Gibson et al., 2007). EtOH is perhaps one of the most overlooked stressors of yeast, especially in the fermented beverage industry where the EtOH is a desired end product. However, EtOH is a toxic metabolic waste product produced by the yeast cells. Despite being one of the most EtOH-tolerant organisms known (Casey and Ingledew, 1986), the increasing concentration of EtOH produced during fermentation hinders the growth (Canetta et al., 2006), viability (Pascual

Abbreviations: EtOH, ethanol; LAB, lactic acid bacteria; AAB, acetic acid bacteria; YPD, yeast extract, peptone, and dextrose; ABV, alcohol by volume.

* Corresponding author.
E-mail addresses: codroger@indiana.edu (C.M. Rogers), doveatch11@gmail.com (D. Veatch), adam@uplandbeer.com (A. Covey), caleb@uplandbeer.com (C. Staton), bochman@indiana.edu (M.L. Bochman).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fm.2016.02.012
0740-0020/

et al., 1988), and fermentative capacity (Fernandes et al., 1997) of *S. cerevisiae*.

Organic acid stress from the lactic acid produced by LAB and acetic acid produced by AAB is also particularly germane to sour beers, and a rich literature exists concerning their effects on *S. cerevisiae* (Ingledew, 2009). Of these two compounds, lactic acid is known to be more detrimental to yeast fermentation (Narendranath et al., 2001a, b; Thomas et al., 2002). Unfortunately, there is no consensus over what is considered an inhibitory concentration of these acids. This is likely due to experimental variations from laboratory to laboratory, as well as the inherent differences in the physiology of the many laboratory and industrial yeast strains that have been investigated. Nevertheless, general guidelines suggest that >0.8% lactic acid and >0.05% acetic acid, as well as pH in the 3.0–4.0 range, should be avoided for maximal fermentation efficiency (Ingledew, 1999).

Although many low-alcohol by volume (ABV) pale beers are packaged immediately after primary fermentation, other styles of beer are racked away from the wort trub and yeast sediment in the bottom of the primary fermentor and allowed to condition in a secondary fermentor or final packaging vessel (bottle, cask, or keg) (Derdelinckx et al., 1992). In a secondary fermentor, this conditioning period affects the flavor and mouthfeel of the beer, as well as aids in the flocculation of suspended yeast cells and high molecular weight compounds (*e.g.*, tannins). When bottle/cask conditioning, this is the period during which the beer is carbonated because a small amount of sugar is added for the resident yeast to metabolize into EtOH and CO_2. This has a negligible effect on the final ABV, but because the bottle is unvented (*i.e.*, capped or corked), the CO_2 produced remains in solution until the bottle is opened. Though essentially any brewing strain of yeast can be used to bottle condition, specialized strains are commercially available that have phenotypic properties suitable for the last stage in beer production, such as a neutral flavor profile and tolerance to high ABV and pressure.

Here, we report the failure of an American sour beer named Cauldron to bottle condition despite the use of a specialized yeast strain. We found that the acidity, especially the concentration of lactic acid, was higher in Cauldron than in similar sours from the same brewery that successfully carbonated via bottle conditioning. The growth medium shock caused by the stressors in Cauldron was characterized, and a protocol was developed to adapt brewing yeast to tolerate these conditions.

2. Materials and methods

2.1. Brewery and beer

The sour beers analyzed herein were brewed by the Upland Brewing Company (http://uplandbeer.com/) in Bloomington, IN, USA. This brewery, opened in 1998, is one of the largest in the American Midwest, and had been brewing sour beers since March of 2006. The Upland sour ale called Cauldron is a 1:1 blend of a Flanders-style red ale and the Dantalion dark wild American ale. The beers were blended post-fermentation after aging for ≥8 months and then further aged in a 265-L oak barrel with 102 kg Michigan Montmorency cherries (~0.6 kg cherries/L of finished beer) for 3 months.

2.2. Strains, media, and other reagents

The following *S. cerevisiae* strains were used: CBC-1, WLP001, WLP300, WLP715, WY1056, and WY2007 (see Table 1 for physiological characteristics and vendor details). All strains were stored as 15% (v/v) glycerol stocks, revived by streaking for single colonies 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 grown in YPD liquid culture at 30 °C with aeration unless otherwise noted. Media components were from Fisher Scientific (Pittsburgh, PA, USA) and DOT Scientific (Burnton, MI, USA). Lactic acid was purchased from DOT Scientific, and 200-proof EtOH was purchased from Pharmco-AAPER (Brookfield, CT, USA). All other reagents were of the highest grade commercially available.

2.3. Chemical analysis of sour beers

Chemical analyses were performed by Brewing & Distilling Analytical Services, LLC (Lexington, KY, USA) on 355-mL samples of the sour beers listed in Table 2 and Supplemental Table 1.

2.4. Methylene blue staining

Cell viability was measured via methylene blue staining. Briefly, yeast cultures were diluted with sterile water, mixed with an equal volume of stain (0.01% (w/v) methylene blue and 2% (w/v) sodium citrate), incubated at room temperature for approximately 60 s, and then observed by light microscopy using an OMAX Model M8311 trinocular compound Siedentopf microscope and 5-MP A1550 digital camera. Images were captured using AmScope version 3.7 software. Cell viability was determined by observing ≥400 cells per condition and calculated as the number cells that excluded the stain divided by the total number of cells. For cells grown in media with a pH < 4, methylene blue staining was unreliable, which is discussed in Section 4.3. For such samples, we report the "apparent viability" as judged by methylene blue staining in Supplemental Fig. 1 and the actual viability (measured by cell counting with a hemocytometer and comparing the total number of cells to the number of colonies formed on YPD agar) in Table 3.

2.5. Growth curves

The yeast strains were grown by inoculating 5 mL YPD medium with single colonies from YPD plates and incubation overnight at 30 °C with aeration in a Fisher Scientific Tube Rotator at 80 rpm. The optical density at 660 nm (OD_{660}) of each culture was determined using a Beckman Coulter DU730 UV/Vis Spectrophotometer. Then, for the experiments in Fig. 2, the cells were diluted to an $OD_{660} = 0.1$ in 200 μL of medium in a round-bottom 96-well plate, overlaid with 50 μL of mineral oil to prevent evaporation, and incubated at 30 °C with linear shaking at 1096 cycles/min in a BioTek Synergy H1 plate reader. The OD_{660} of every well was measured and recorded every 15 min for ~24 h, and these values were plotted *vs.* time to generate growth curves. For the experiments in Table 4 and Supplemental Fig. 2, the cells were grown as described above but in the absence of aeration to better mimic bottle conditioning. Thus, to obtain an accurate OD_{660} reading, the cells were manually resuspended in the 96-well plate using a micropipettor prior to measuring the OD_{660} in the plate reader. As such, fewer time points were recorded. Regardless, all growth experiments were repeated ≥3 times, and the plotted values represent the average ± standard deviation.

2.6. Carbonation assays

A standard bottle conditioning method is employed by the Upland Brewery. Briefly, glucose was dissolved in sterile water and added to the beer to a final concentration of 0.012 g/mL, which is approximately equal to 1.25% (w/v). In most cases, dry CBC-1 yeast (Lallemand, Montreal, QC, Canada) was used. Ten micrograms of dry yeast per milliliter of beer to be carbonated (approximately 1–2

Table 1
Yeast strains used.

Strain	Common name	Attenuation	Flocculation	Temperature optimum	Alcohol tolerance	Source[b]
CBC-1	Cask & Bottle Conditioned Beer Yeast	N.D.[a]	High	15–25 °C	12-14% ABV	Lallemand
WLP001	California Ale Yeast	73–80%	Medium	20–23 °C	High	White Labs
WLP300	Hefeweizen Ale Yeast	72–76%	Low	20–22 °C	Medium	White Labs
WLP715	Champagne Yeast	>75%	Low	21–24 °C	17% ABV	White Labs
WY1056	American Ale Yeast	73–77%	Medium-low	15–22 °C	11% ABV	Wyeast Laboratories
WY2007	Pilsen Lager Yeast	71–75%	Medium	9–13 °C	9% ABV	Wyeast Laboratories

[a] Abbreviations: N.D., not determined; and ABV, alcohol by volume.
[b] Lallemand (Montreal, QC, Canada), http://www.danstaryeast.com; White Labs (San Diego, CA, USA), www.whitelabs.com; and Wyeast Laboratories (Odell, OR, USA), www.wyeastlab.com.

Table 2
Characteristics of sour beers that did not bottle condition and average characteristics of those that did.

Style	ABV[a] (% at 20 °C)	SG	OG	Fermentation (%)	Bitterness (IBU)	Color (SRM)	pH	Total acidity (% lactic acid)	Volatile acidity (% acetic acid)
Blueberry Lambic[b]	6.52	1.00986	N.D.	68.54	8.0	22.5	2.95	2.540	N.D.
Blueberry Lambic	6.97	1.00776	N.D.	71.70	9.0	20.6	3.08	2.380	N.D.
Cauldron	8.37	1.01976	1.08258	62.99	10.0	35.69	3.17	2.50	0.25
Carbonated sours[c]	6.84 (4.81–9.78)	1.01080 (1.00538–1.01293)	1.06335 (1.0854–1.0422)	68.21 (64.96–74.46)	8.22 (5–13)	16.23 (3–59.44)	3.23 (3.02–3.47)	1.429 (0.99–1.63)	0.17 (0.15–0.21)

[a] Abbreviations: ABV, alcohol by volume; SG, specific gravity; OG, original gravity; IBU, international bittering units; SRM, standard reference method; and N.D., not determined.
[b] The values shown for the non-carbonated sours (Blueberry Lambics and Cauldron) in this table are the average of ≥3 measurements.
[c] The values shown for the Carbonated sours in this table are averages (and ranges) of triplicate data from 13 different sour beers. See Supplemental Table 1 for the full dataset.

Table 3
The effects of growth medium pH on yeast cell size and viability.

Growth medium	pH	Cell size (μm)	Viability[a]
Water	7.2	2.68 ± 0.68	68.8 ± 10.4%
Cauldron	3.17	2.77 ± 0.75	79.8 ± 6.42%
YPD	7	5.77 ± 0.93	92.3 ± 1.94%
YPD	6	5.36 ± 0.60	94.5 ± 1.66%
YPD	5	5.20 ± 0.92	94.4 ± 2.40%
YPD	4	4.09 ± 1.41	95.5 ± 0.12%
YPD	3	4.00 ± 1.40	94.6 ± 1.07%
YPD	2.5	2.95 ± 0.61	75.8 ± 6.11%

[a] Viability was measured by methylene blue staining and/or counting cells with a hemocytometer and comparing the total number of cells to colonies formed by plating on YPD agar.

million cells/mL) were rehydrated in 10 mL sterile distilled water per gram of yeast at 37 °C for 15 min, gently stirred, and then incubated for an additional 5 min. This slurry was then used to inoculate the glucose-containing beer, and the beer was mixed and bottled. Thus, the final inoculant equates to 0.75–1.5 billion cells/750 mL bottle. In beers that carbonated (Table 2 and Supplemental Table 1), this procedure resulted in the production of ~4 volumes of CO_2 in solution for every bottle. The Upland Brewery uses 750-mL glass champagne-style bottles stoppered with a cork that is wired in place; experiments in the laboratory used standard 12-oz beer bottles capped with O_2-absorbing bottle caps (Midwest Supplies, Minneapolis, MN, USA). Multiple modified protocols were attempted to carbonate Cauldron, which was recalcitrant to bottle conditioning as described above, and these are detailed in Subsections 3.4 and 3.6 of the Results. Images of the beers that underwent bottle conditioning attempts were captured with the digital camera of a Samsung Galaxy S5 smart phone mounted onto a tripod using a custom 3D-printed bracket based on a design found at http://contractorwolf.com/3d-printed-phone-tripod-mount/. The editable.skp files and printable.stl files are included as supplemental material and can be opened and manipulated using the freely available program SketchUp (http://www.sketchup.com/). All bottling experiments were repeated ≥3 times, and representative results are presented.

2.7. Statistical analyses

Student's t-tests (unpaired) were used to compare data using GraphPad Prism 6, and p-values <0.05 were considered significant.

3. Results

3.1. Chemical analysis comparing beers that carbonated with those that did not

Commercial laboratory analysis was used to compare 16 batches of beer that carbonated with three that did not. As can be seen in Table 2 and Supplemental Table 1, there was very little difference between these two groups for all of the variables assessed, including ABV. However, the pH and the total acidity (as the percent of lactic acid) differed significantly between the beers that carbonated and those that did not (p = 0.048 and 0.000052, respectively) (Fig. 1). Thus, the beers that failed to bottle condition contained more lactic acid, which decreased the pH, relative to the beers that did properly carbonate. Cauldron also contained more acetic acid (0.25% of volatile acidity, Table 2 and Supplemental Table 1) than the beers that carbonated (0.09–0.21%), but insufficient data exists to determine the significance of this observation.

3.2. The yeast did not die during bottling

We hypothesized that Cauldron failed to carbonate because the low pH/high [lactic acid] was killing the cells. To investigate this, we performed vital staining of both CBC-1 cells that were rehydrated via the same protocol used to prepare the yeast for bottle

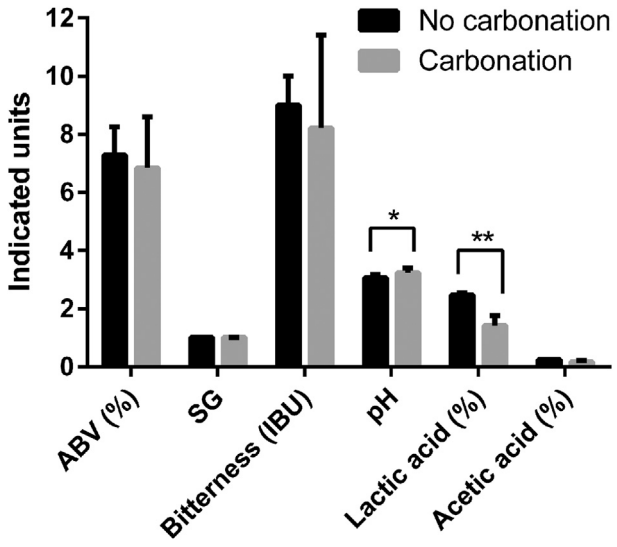

Fig. 1. Comparison of beers that carbonated *vs.* those that did not. The indicated data from Table 2 and Supplemental Table 1 were averaged for the 16 beers that carbonated by bottle conditioning and the three that did not. The pH, mostly due to the high concentration of lactic acid, represents the largest difference between the carbonated and non-carbonated beers. The plotted values are the means, and the error bars are the standard deviations. $^{*}p < 0.05$, $^{**}p < 0.01$.

conditioning and cells recovered from bottles that had been exposed to Cauldron for 2 weeks (Supplemental Fig. 1 and Table 3). We found that nearly one-third of rehydrated CBC-1 cells were dead (68.8 ± 10.4% viability), unlike CBC-1 cells revived from glycerol stocks in YPD medium (92.3 ± 1.94% viability). Further, the rehydrated cells were smaller (2.68 ± 0.68 *vs.* 5.77 ± 0.93 μm, $p = 0.012$) and unbudded (Supplemental Fig. 1B), consistent with cells that have not re-entered the cell cycle and begun to grow yet. When we observed CBC-1 cells after incubation in Cauldron for 2 weeks, they were also significantly smaller (2.77 ± 0.75 μm, $p = 0.012$) and unbudded relative to cells recovered in YPD medium (Supplemental Fig. 1C). Contrary to our expectations, however, methylene blue staining indicated that the majority of the cells were alive (85.0% apparent viability, see Sections 2.4 and 4.3). Subsequently plating these cells on YPD agar also demonstrated their viability (79.8 ± 6.42% of recovered cells were viable, Table 3), as colonies formed within 2 days of incubation at 30 °C (data not shown).

3.3. Lactic acid stress differentially affects brewing strains

We next investigated the possibility that the high levels of lactic acid in Cauldron resulted in a growth medium shock from which the CBC-1 cells did not recover. To do this, we cultured the yeast in YPD medium at pH 7 (YPD-pH7), back diluted into either YPD-pH7 or YPD medium that had been acidified using lactic acid (YPD-pH6, -pH5, -pH4, and -pH3), and followed the growth of each culture for 24 h. As shown in Fig. 2A, CBC-1 grew well under all conditions, with only YPD-pH3 medium resulting in a slight decrease in the maximum growth rate (4.88 ± 0.97 *vs.* 5.53 ± 0.95; calculated as the maximum OD_{660}/time) and time to reach the maximum growth rate (481 ± 1.44 min *vs.* 345 ± 1.08 min) relative to all of the other media. Vital staining and microscopic examination of cells grown in YPD medium at different pH's yielded results consistent with the growth curves (Supplemental Fig. 1A, D-G and Table 3). It should be noted that the YPD-pH3 medium is similar to Cauldron (pH 3.17), but cells incubated in YPD-pH3 medium were larger on average and displayed more buds than the cells in Cauldron (Table 3 and Supplemental Fig. 1C,G). Possible reasons for these differences are discussed in Section 4.2.

Although the YPD-pH3 medium approximated the acidity of Cauldron, the growth of CBC-1 was only mildly affected, making it unlikely that this was the cause of the defect in bottle conditioning. However, this remained a formal possibility. Thus, we performed similar growth assays as above with other common brewing strains (Table 1) in an attempt to identify one or more that were resistant to low pH/high [lactic acid] conditions. To our surprise, the growth curves of each strain displayed highly variable trends depending on the pH of the growth medium (Fig. 2). For instance, the growth rate of the ale strains WLP001 and WY1056 tended to decrease with decreasing pH (Fig. 2B and C), the growth of the lager strain WY2007 and hefeweizen strain WLP300 were only affected by the lowest pH media (Fig. 2D and E), and pH only affected the lag time but not growth rate of the champagne yeast WLP715 (Fig. 2F).

3.4. Healthy CBC-1 and champagne yeast did not carbonate Cauldron

The above experiments indicated that the CBC-1 strain can grow in low pH media. However, unlike the cells used to attempt to bottle condition Cauldron in the brewery, those used to generate that data in Fig. 2A came from a healthy culture growing in rich medium rather than desiccated yeast that was rehydrated immediately before addition to the beer. It is well known that desiccation and subsequent rehydration are major stressors of yeast that can cause cellular damage and affect fermentation performance (reviewed in (Gibson et al., 2007)), but the daughter cells of the dehydrated/rehydrated yeast do not display these defects. Thus, CBC-1 may have tolerated the YPD-pH3 medium because it was given a chance to recover from desiccation prior to experiencing an acidic shock. To investigate this, we performed two carbonation experiments. In the first, we rehydrated CBC-1 cells using the standard brewery procedure, streaked those cells for single colonies on YPD-pH7 agar plates, and then grew the cells to high density in liquid YPD-pH7 medium. These cultures were used to inoculate 350 mL of Cauldron containing 1.25% glucose with 2×10^6 cells/mL CBC-1 in glass bottles, which were then incubated at ambient temperature (23–25 °C) for 2 weeks. In the second control experiment, CBC-1 cells were rehydrated using the standard brewery procedure and used immediately to inoculate Cauldron. After 2 weeks of incubation, the control bottles failed to carbonate, recapitulating the results of the brewery (Fig. 3A). Unfortunately, the bottles inoculated with healthy CBC-1 also failed to carbonate (data not shown).

Of all of the yeast strains examined in Fig. 2, only the WLP715 champagne strain displayed better growth kinetics in low pH/high [lactic acid] media than CBC-1 (Fig. 2F). Though subtle in the context of growth curves, perhaps this more robust growth would make WLP715 a better choice for bottle conditioning. Indeed, as shown in Table 1, WLP715 is a hardy strain known for its high levels of attenuation and alcohol tolerance. Therefore, we tested its ability to carbonate Cauldron by inoculating the beer with 2×10^6 cells/mL WLP715 and incubating as described above. However, upon uncapping the bottles, no carbonation was evident (Fig. 3B), and further incubation for an additional 2 weeks had no effect (data not shown).

3.5. Terminal acidic shock inhibits sour beer bottle conditioning

Stymied by our repeated failures to carbonate Cauldron and lacking the base wort that Cauldron was fermented from, we attempted to alter our experimental conditions by adding 8% (v/v) EtOH to our YPD medium to better mimic the beer. EtOH becomes increasingly toxic to yeast cells as its concentration increases

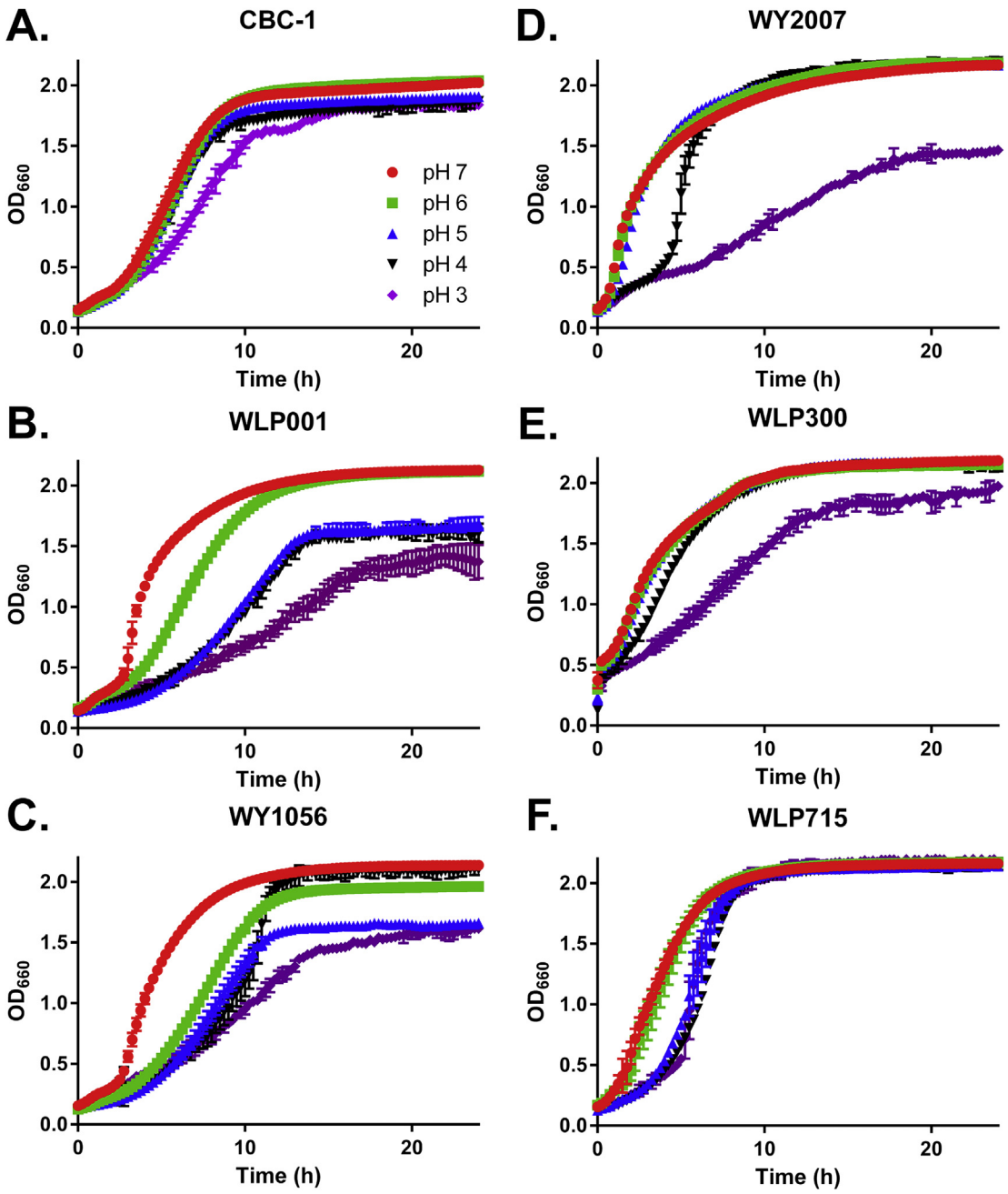

Fig. 2. The effects of lactic acid on brewer's yeast growth kinetics. The indicated strains were grown to saturation overnight in YPD medium, back-diluted into YPD medium lacking lactic acid (pH 7) or containing increasing amounts of lactic acid (pH 6, 5, 4, and 3), and their growth was followed via OD_{660} measurements to plot growth curves. Strains CBC-1 and WLP715 were least affected by changes in pH (**A** and **F**, respectively). The remaining strains (**B-E**) displayed decreased growth kinetics with increasing acidity, especially at pH 3. All experiments were performed ≥3 times on biological replicates. The data shown are averages, and the error bars are the standard deviation.

Table 4
The effects of lactic acid and ethanol (EtOH) on CBC-1 growth.

Growth medium	Starting OD_{660}	OD_{660} at 4 h	OD_{660} at 19 h
YPD-pH7	0.123 ± 0.008	0.355 ± 0.007	1.57 ± 0.021
YPD-pH7 + EtOH	0.107 ± 0.014	0.166 ± 0.005	1.44 ± 0.042
YPD-pH3	0.167 ± 0.036	0.269 ± 0.037	1.58 ± 0.032
YPD-pH3 + EtOH	0.194 ± 0.061	0.184 ± 0.058	1.16 ± 0.064
YPD-pH2.5	0.117 ± 0.008	0.114 ± 0.013	0.128 ± 0.016
YPD-pH2.5 + EtOH	0.133 ± 0.006	0.147 ± 0.036	0.127 ± 0.020

during fermentation (Briggs et al., 2004), and the average ABV in American craft beers in <6% (Nelson, 2014). Thus, we reasoned that the high EtOH content of Cauldron (8.37%) in combination with its low pH may have inhibited bottle conditioning. To test this, we grew CBC-1 in YPD medium at various pH's as in Supplemental Fig. 1 with and without the addition of 8% EtOH. As bottle conditioning is also an anaerobic process, these cultures were not aerated. At neutral pH, the addition of EtOH increased the lag time of the culture by > 1 h (Table 4 and Supplemental Fig. 2). In YPD-pH3 medium, the presence of EtOH increased the lag time by > 1 h, decreased the maximum growth rate by 75%, and decreased the maximum density of the culture by 0.24 OD_{660} (approximately 3 million fewer cells per milliliter). To our surprise, no growth was observed in YPD-pH2.5 medium, regardless of the

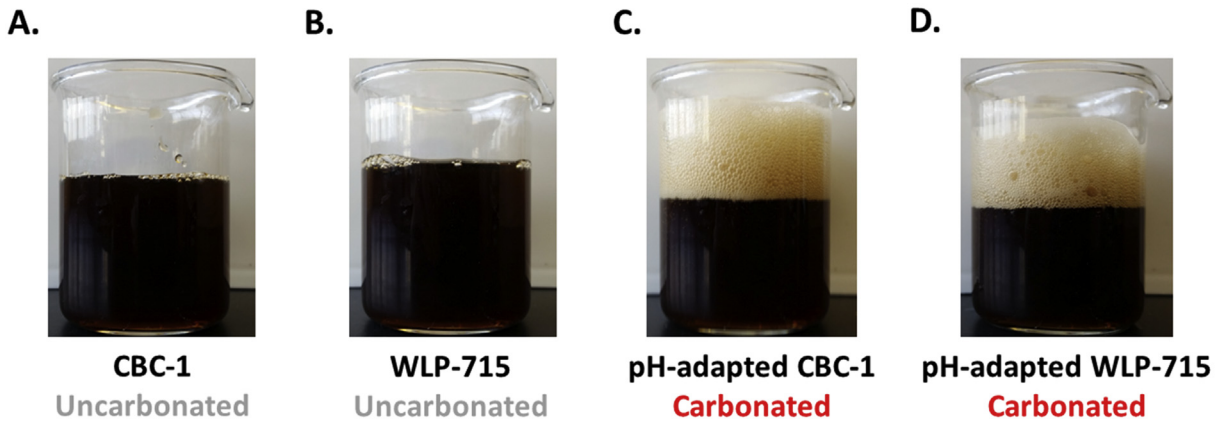

Fig. 3. CBC-1 and WLP715 fail to bottle condition Cauldron using conventional methods. (**A**) CBC-1 and (**B**) WLP-715 did not carbonate Cauldron during 2 weeks of bottle conditioning. However, if these strains were pre-grown in a 1:1 mixture of YPD-pH7 medium and Cauldron (pH adapted; **C** and **D**), bottle conditioning was successful.

presence of EtOH. Methylene blue staining suggested that at least half of these cells were still viable (Supplemental Fig. 1H), though determining viability was difficult because the cells formed tight clumps and methylene blue staining was poor at low pH (see Sections 2.4 and 4.3). However, colony counting on YPD agar verified that the viability of the cells grown in YPD-pH2.5 medium was >75% (Table 3). Regardless, all of the cells were small and unbudded (as was a large fraction of the cells grown in YPD-pH3 medium; Supplemental Fig. 1G–H), indicating that they were not actively growing in the YPD-pH2.5 medium.

Thus, the pH of Cauldron (pH = 3.17; Table 2) is close to the limit of that tolerated by CBC-1 cells for growth. Together with all of the results above, this suggests that the combination of low pH, high [EtOH], lack of O_2, and perhaps other stresses in Cauldron (*e.g.*, limiting nutrients and acetic acid) resulted in a condition that we have dubbed "terminal acidic shock" from which the yeast cannot recover when inoculated into Cauldron. The cells likely cease active growth and enter the G_0 phase of the cell cycle (stationary phase or quiescence) (Herman, 2002), preventing bottle conditioning.

3.6. *S. cerevisiae* can be adapted to overcome terminal acidic shock conditions

Microbes are amazingly resilient and can adapt to growth in changing environmental conditions. Indeed, *S. cerevisiae* can be adapted to tolerate toxins such as heavy metals (Minney and Quirk, 1985) and EtOH (Dinh et al., 2008). As such we sought to adapt CBC-1 to growth in Cauldron prior to using the strain for bottle conditioning. Fresh colonies from YPD agar plates were inoculated into YPD medium and grown to saturation overnight with aeration at 30 °C. Then, these cells were used to inoculate YPD-pH7, YPD-pH3, YPD-pH3 supplemented with 8% EtOH (YPD-pH3 + EtOH), or a combination of YPD-pH7 and Cauldron mixed in a 1:1 ratio (YPD-Cauldron) media, and the cultures were incubated at 30 °C with aeration. As expected from Table 3 and Supplemental Figs 1 and 2, the cells in YPD-pH7 and YPD-pH3 media grew to saturation overnight (~18 h), but those in YPD-pH3 + EtOH medium required slightly longer (up to 24 h) (data not shown). The cells in YPD-Cauldron medium, however, required 36–48 h to reach saturation.

Next, cells from each culture were added to the same density to 12-oz bottles containing Cauldron and 1.25% glucose. The bottles were tightly capped, their contents mixed by inversion, and stored at ambient temperature. After 2 weeks, the bottles inoculated with CBC-1 grown in YPD-pH7, YPD-pH3, and YPD-pH3 + EtOH media had failed to carbonate (data not shown). However, the beer inoculated with CBC-1 grown in YPD-Cauldron medium did properly bottle condition, resulting in audible degassing upon removal of the cap and a thick head of foam on the beer when poured (Fig. 3C). Interestingly, after an additional 2 weeks of incubation, bottles inoculated with CBC-1 grown in either YPD-pH3 or YPD-pH3 + EtOH medium were also found to have carbonated (data not shown). Thus, CBC-1 could be adapted to bottle condition Cauldron by pre-growing the cells in a medium that partially recapitulates the Caldron milieu.

To determine if this adaptation was specific to the CBC-1 strain, we repeated the above experiments using WLP715. As with CBC-1, the WLP715 cells displayed similar growth kinetics in YPD-pH7, YPD-pH3, YPD-pH3 + EtOH, and YPD-Cauldron media (data not shown). Similarly, all bottles failed to carbonate after 2 weeks except those that were inoculated with cells pre-grown in YPD-Cauldron medium (Fig. 3D). As described above, however, WLP715 pre-grown in YPD-pH3 or YPD-pH3 + EtOH medium was able to bottle condition Cauldron after 4 weeks of incubation (data not shown).

4. Discussion

Our analysis of Cauldron and the growth of brewer's yeast as affected by pH and EtOH revealed a type of stress response, terminal acidic shock, that *S. cerevisiae* can undergo when exposed to the harsh environmental conditions of an extreme American sour beer. We found that terminal acidic shock was not lethal to the cells but, rather, inhibited their metabolism. Further, the yeast could be adapted to growth in these acidic conditions, resulting in carbonation of the beer while bottled. Our protocol is currently being scaled up for use with the next batch of Cauldron that is being produced.

4.1. The effects of pH on fermentation should not be underestimated

As stated above, there is a long history of brewers and researchers studying the effects of pH on yeast EtOH fermentation, but most of this literature is rife with inconsistencies and conflicting data. Unfortunately, the results presented here only add to the list of discrepancies. For instance, we found that *S. cerevisiae* strain CBC-1 did not grow in rich medium at pH = 2.5, but growth under such acidic conditions has previously been reported (Carmelo et al., 1996). The differences between these datasets may be due to the fact that we investigated pH effects on industrial strains of *S. cerevisiae*, while Carmelo et al. (1996) used common

laboratory strains (YPH499 genetic background) (Sikorski and Hieter, 1989). Alternatively, our results may differ from other published reports due to the acid used (lactic acid here *vs*. HCl in (Carmelo et al., 1996)) because various organic and inorganic acids differentially affect yeast growth (Narendranath et al., 2001a, b; Thomas et al., 2002). Regardless, as shown in Fig. 1, small changes in pH should not be ignored when analyzing beer because they can greatly affect the final product. Quality control and quality assurance personnel in brewery laboratories would be wise to remember that pH is a log-based scale, and thus even minor differences in pH (3.07 ± 0.11 for non-carbonated *vs*. 3.23 ± 0.16 for carbonated beer) can be significant differences in the molar concentration of the acid (1.44-fold here), especially in sour beers containing organic acids such as lactic acid.

It was also surprising to find that all of the industrial strains analyzed yielded different growth profiles in media with decreasing pH (Fig. 2). Some strains were relatively unaffected by drops in pH by up to three pH units (1000-fold molar difference), while others displayed appreciable decreases in growth rate comparing growth in pH 7 *vs*. pH 6 media (*e.g*., WY1056). We hypothesize that these differences are due to genetic variation among the different *S. cerevisiae* isolates. It has recently been shown that >100 genes significantly contribute to lactic acid stress adaptation by *S. cerevisiae* (Suzuki et al., 2012), and even single nucleotide polymorphisms in one or more of these genes could result in differential lactic acid sensitivity. Lacking full genome sequences for all of the strains used, however, precludes us from investigating this hypothesis. Regardless, by pH 3, all strains displayed diminished growth kinetics and failed to carbonate Cauldron (Fig. 3 and data not shown), so terminal acidic shock likely has a common genetic or physiological determinant in these strains.

4.2. Stressors other than pH

As shown in Table 2 and Supplemental Table 1, Cauldron was not unique in failing to bottle condition; both Blueberry Lambics also remained uncarbonated. They had lower ABVs than Cauldron, which should decrease the stress that the bottle conditioning yeast experienced. However, they were both more acidic than Cauldron ($pH \leq 3.08$ *vs*. 3.17), which likely terminally shocked the CBC-1 cells. Unfortunately, lacking samples of the uncarbonated Blueberry Lambics, we cannot directly test this. Regardless, even acidifying YPD medium with lactic acid and adding EtOH to adapt CBC-1 and WLP750 cells to bottle condition Cauldron only partially recreated the chemical composition of the beer. This is evident in the fact that YPD-Cauldron medium was more effective to "prime" the yeast for bottle conditioning than YPD-pH3 ± EtOH medium (Fig. 3 and data not shown).

The most parsimonious explanation for this is that Cauldron contains stressors in addition to lactic acid and EtOH that also inhibit bottle conditioning. The culprit could be acetic acid, which was also present in the Blueberry Lambics due to the AAB included during their primary fermentations (though the % acetic acid was not determined in the Blueberry Lambics, see Table 2 and Supplemental Table 1). Indeed, much like lactic acid, increasing concentrations of acetic acid decrease yeast growth (Narendranath et al., 2001a, b; Thomas et al., 2002). However, other phytochemicals extracted from the cherries in Cauldron and blueberries in the Lambics could also be responsible (*e.g*., phenolics like tannins) (Skrovankova et al., 2015). Water and EtOH are both excellent solvents capable of extracting such compounds, which are known to have antimicrobial activities (see (Joshi et al., 2014; Lacombe et al., 2013) and references therein).

These other stressors may also be why cells incubated in Cauldron were small (Table 3) and unbudded when observed by microscopy (Supplemental Fig. 1C) relative to cells incubated in YPD-pH3 medium (Supplemental Fig. 1G). The low-oxygen, low-nutrient, high-EtOH, acidic, complex chemical makeup of Cauldron cannot be completely duplicated in the lab to determine the relative effects of individual stressors on the yeast. Thankfully, simply mixing Cauldron with rich medium was enough to both maintain active metabolism by the yeast and "prime" them for bottle conditioning under the harsher 100% Cauldron conditions (Fig. 3C,D).

We hypothesize that diluting the Cauldron stressors by half in YPD-Cauldron medium, as well as growing the cells with aeration and reintroducing nutrients, is sufficient to generate a population of healthy cells that have adapted to tolerate the bioactive chemicals in Cauldron. Similarly, adapting the cells to a significant Cauldron stressor (*e.g*., lactic acid) by growing them in YPD-pH3 medium also yielded cells capable of bottle conditioning the beer. These cells were not completely adapted to the Cauldron milieu as they took twice as long to bottle condition the beer as cells grown in YPD-Cauldron medium, but our hypothesis remains the same. Thus, pre-adapting yeast in a mixture of rich medium and beer may be a general method to ensure proper bottle conditioning of any beer.

4.3. Dry yeast should be used with caution for "extreme" beers

Prior to the 1990s, when the American craft beer revolution was in its infancy, dry yeast was the only option for the majority of home brewers and professional brewers who lacked the expertise and equipment necessary for liquid yeast propagation (Dornbusch and Mott, 2006). The dry yeast available was inconsistent, resulting in random fermentation kinetics from lot to lot, and occasionally included unwanted bacteria or spoilage yeasts. The emergence of companies that specialize in producing and selling high quality liquid yeast cultures changed all of that, and until recently, liquid brewer's yeast was considered superior to dry yeast. However, these same companies have since begun to reintroduce dry yeast to brewers, touting its lower cost, longer shelf life, and the fact that starter cultures are not needed to achieve a particular pitch rate (*i.e*., inoculation density) (Carpenter, 2014). In side-by-side tests, dry yeast is also often found to be equivalent to liquid culture for brewing (Dornbusch and Mott, 2006), but these results should be interpreted with caution.

We found that the viability of rehydrated CBC-1 yeast was <70% (Table 3), and the drying process itself is harsh (reviewed in (Gibson et al., 2007)). The desiccation and subsequent rehydration of yeast prior to addition to beer damages the yeast cells, likely accounting for the non-viable CBC-1 cells, and leaves the viable cells vulnerable to the harmful effects of the beer (*e.g*., ethanol and/or pH stress). For a typical craft beer with <6% ABV (Nelson, 2014) and a pH of ~4.1 from carbonic acid production via CO_2 (Coote and Kirsop, 1976), the rehydrated yeast can tolerate these mild stressors and thrive to bottle condition the beer. For a sour beer like Cauldron, with a greater than average ABV (8.37%, Table 2) and containing additional organic acids, terminal acidic shock occurs, and bottle conditioning is disrupted.

4.4. Methylene blue staining of yeast is not reliable at low pH

As with beer pH, the literature concerning methylene blue staining and the effects of various conditions on staining efficiency is abundant and full of contradictory findings (reviewed in (Arthur and Shelley, 1959)). For instance, some reports claim that low pH yields better and more intense blue staining of dead cells, while others describe just the opposite. We found that $pH < 4$ interferes with methylene blue staining of yeast (Table 3 and Supplemental Fig. 1). Under such conditions, some cells appeared to have taken

up slightly more dye than others, but the scoring was difficult because the intense blue staining evident at higher pH was not observed. Thus, viability data are reported as "apparent viability" (*i.e.*, total cells - darker cells/total cells) for cells grown under these conditions rather than the percent of viable cells as for cells grown in less acidic media.

It is unclear if this is an effect of the pH on the chemistry of the methylene blue stain itself or a side effect of yeast physiology at low pH. On one hand, pH is known to change the ionization state of methylene blue, which alters the intensity of staining (Arthur and Shelley, 1959). On the other hand, pH also affects flocculation in yeast, maximally increasing it between pH 2.5 and 4.5 (Helm et al., 1953). We did observe an increasing tendency of the CBC-1 cells to clump together as the pH decreased (Supplemental Fig. 1 and data not shown), which is particularly evident for this strain at pH 3 and 2.5 (Supplemental Fig. 1G–H).

Regardless, methylene blue is widely utilized in the quality control laboratories of many breweries due to its ease of use and low expense. Based on the data presented here, we would caution such laboratories against using methylene blue staining to determine cell viability in beers of very low pH. Instead, colony counting of serial dilutions on plating medium (*e.g.* YPD agar) is recommended. This method is slower, but the results will be more accurate.

5. Conclusions

All beers are complex mixtures of chemicals derived from the base grains, hops, yeasts, and/or adjuncts from which they are brewed. Thus, we are hesitant to blame the bottle conditioning problems experienced with Cauldron beer and CBC-1 yeast on a single variable such as EtOH or lactic acid. Indeed, CBC-1 cells pre-grown in YPD-Cauldron medium were able to carbonate Cauldron in a shorter time frame than cells pre-grown in YPD-pH3 medium acidified with lactic acid. Regardless, even partially recapitulating the stressors found in Cauldron was enough to adapt to cells for successful bottle conditioning. Therefore, we recommend that craft brewers allow their bottle conditioning strains time to adapt to the beer that they are trying to carbonate prior to bottling.

Acknowledgments

We thank members of the Bochman and van Kessel laboratories for critically reading and providing feedback on this manuscript. This work was supported by startup funds from Indiana University and a Translational Research Pilot Grant from the Johnson Center for Entrepreneurship in Biotechnology (to MLB).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.02.012.

References

Arthur, R.P., Shelley, W.B., 1959. The technology of in vitro staining of nerves in human skin with thiazin dyes. J. Invest Dermatol 33, 121–144.
Briggs, D., Boulton, C., Brookes, P., Stevens, R., 2004. Brewing: Science and Practice. Woodhead, Cambridge, UK.
Canetta, E., Adya, A.K., Walker, G.M., 2006. Atomic force microscopic study of the effects of ethanol on yeast cell surface morphology. FEMS Microbiol. Lett. 255, 308–315.
Carmelo, V., Bogaerts, P., Sa-Correia, I., 1996. Activity of plasma membrane H+-ATPase and expression of PMA1 and PMA2 genes in Saccharomyces cerevisiae cells grown at optimal and low pH. Arch. Microbiol. 166, 315–320.
Carpenter, D., 2014. Liquid vs. Dry yeast. In: Craft Beer & Brewing. http://beerandbrewing.com/VKRJ0isAAF-R9GMY/article/liquid-vs.-dry-yeast: Unfiltered Media Group,LLC.
Casey, G.P., Ingledew, W.M., 1986. Ethanol tolerance in yeasts. CRC Crit. Rev. Microbiol. 13, 219–280.
Coote, H., Kirsop, B.H., 1976. Factors responsible for the decrease in pH during beer fermentations. J. Inst. Brew. 82, 149–153.
De Keermaecker, J., 1996. The mystery of lambic beer. In: Scientific American, pp. 74–81.
Derdelinckx, G., Vanderhasselt, B., Maudoux, M., Dufour, J.P., 1992. Refermentation in bottles and kegs: a rigorous approach. Brauwelt Int. 2, 156.
Dinh, T.N., Nagahisa, K., Hirasawa, T., Furusawa, C., Shimizu, H., 2008. Adaptation of Saccharomyces cerevisiae cells to high ethanol concentration and changes in fatty acid composition of membrane and cell size. PLoS One 3, e2623.
Dornbusch, H., Mott, T., 2006. Liquid versus dried yeast: an old debate revisited. Zymurgy 30–36.
Fernandes, L., Corte-Real, M., Loureiro, V., Loureiro-Dias, M.C., Leao, C., 1997. Glucose respiration and fermentation in Zygosaccharomyces bailii and Saccharomyces cerevisiae express different sensitivity patterns to ethanol and acetic acid. Lett. Appl. Microbiol. 25, 249–253.
Gibson, B.R., Lawrence, S.J., Leclaire, J.P., Powell, C.D., Smart, K.A., 2007. Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31, 535–569.
Helm, E., Nohr, B., Thorne, R.S.W., 1953. The measurement of yeast flocculence and its significance in brewing. Wallerstein Commun. 16, 315–325.
Herman, P.K., 2002. Stationary phase in yeast. Curr. Opin. Microbiol. 5, 602–607.
Ingledew, W.M., 1999. Alcohol production by *Saccharomyces cerevisiae*: a yeast primer. In: The Alcohol Textbook: a Reference for the Beverage, Fuel and Industrial Alcohol Industries. Nottingham University Press, Nottingham, UK, pp. 49–87.
Ingledew, W.M., 2009. Yeast stress in the fermentation process. In: The Alcohol Textbook. Nottingham University Press, Nottingham, UK, pp. 115–126.
Joshi, S.S., Howell, A.B., D'Souza, D.H., 2014. Cronobacter sakazakii reduction by blueberry proanthocyanidins. Food Microbiol. 39, 127–131.
Lacombe, A., Tadepalli, S., Hwang, C.A., Wu, V.C., 2013. Phytochemicals in lowbush wild blueberry inactivate Escherichia coli O157:H7 by damaging its cell membrane. Foodborne Pathog. Dis. 10, 944–950.
Li, H., Liu, F., 2015. The chemistry of sour taste and the strategy to reduce the sour taste of beer. Food Chem. 185, 200–204.
Minney, S.F., Quirk, A.V., 1985. Growth and adaptation of Saccharomyces cerevisiae at different cadmium concentrations. Microbios 42, 37–44.
Narendranath, N.V., Thomas, K.C., Ingledew, W.M., 2001a. Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J. Am. Soc. Brew. Chem. 59, 187–194.
Narendranath, N.V., Thomas, K.C., Ingledew, W.M., 2001b. Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. J. Ind. Microbiol. Biotechnol. 26, 171–177.
Nelson, D., 2014. In: Bresswein, K. (Ed.), Craft Beer Brewers' Range of Alcohol Contents Poses Quandry in Preventing Over-serving. PennLive LLC.
Pascual, C., Alonso, A., Garcia, I., Romay, C., Kotyk, A., 1988. Effect of ethanol on glucose transport, key glycolytic enzymes, and proton extrusion in Saccharomyces cerevisiae. Biotechnol. Bioeng. 32, 374–378.
Sikorski, R.S., Hieter, P., 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27.
Skrovankova, S., Sumczynski, D., Mlcek, J., Jurikova, T., Sochor, J., 2015. Bioactive compounds and antioxidant activity in different types of berries. Int. J. Mol. Sci. 16, 24673–24706.
Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2014. The microbial diversity of traditional spontaneously fermented lambic beer. PLoS One 9, e95384.
Suzuki, T., Sugiyama, M., Wakazono, K., Kaneko, Y., Harashima, S., 2012. Lactic-acid stress causes vacuolar fragmentation and impairs intracellular amino-acid homeostasis in Saccharomyces cerevisiae. J. Biosci. Bioeng. 113, 421–430.
Thomas, K.C., Hynes, S.H., Ingledew, W.M., 2002. Influence of medium buffering capacity on inhibition of Saccharomyces cerevisiae growth by acetic and lactic acids. Appl. Environ. Microbiol. 68, 1616–1623.
Tonsmeire, M., 2014. American Sour Beer: Innovative Techniques for Mixed Fermentations. Brewers Publications, Boulder, CO.