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