International Journal of Food Microbiology 206 (2015) 75–80

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Thermal resistance of *Saccharomyces* yeast ascospores in beers

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ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form 16 March 2015
Accepted 3 April 2015
Available online 11 April 2015

Keywords:
Pasteurization
Ethanol
Heat resistance
Strain
Sporulation method
Sporulation rate

ABSTRACT

The industrial production of beer ends with a process of thermal pasteurization. *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* are yeasts used to produce top and bottom fermenting beers, respectively. In this research, first the sporulation rate of 12 *Saccharomyces* strains was studied. Then, the thermal resistance of ascospores of three *S. cerevisiae* strains (DSMZ 1848, DSMZ 70487, Ethanol Red®) and one strain of *S. pastorianus* (ATCC 9080) was determined in 4% (v/v) ethanol lager beer. $D_{60°C}$-values of 11.2, 7.5, 4.6, and 6.0 min and z-values of 11.7, 14.3, 12.4, and 12.7 °C were determined for DSMZ 1848, DSMZ 70487, ATCC 9080, and Ethanol Red®, respectively. Lastly, experiments with 0 and 7% (v/v) beers were carried out to investigate the effect of ethanol content on the thermal resistance of *S. cerevisiae* (DSMZ 1848). $D_{55°C}$-values of 34.2 and 15.3 min were obtained for 0 and 7% beers, respectively, indicating lower thermal resistance in the more alcoholic beer.

These results demonstrate similar spore thermal resistance for different *Saccharomyces* strains and will assist in the design of appropriate thermal pasteurization conditions for preserving beers with different alcohol contents.

1. Introduction

A Sumerian tablet found in Mesopotamia dated 6000 years ago is the oldest evidence of beer production (Mirsky, 2007; Nelson, 2014). Beer is an alcoholic beverage obtained by yeast fermentation of the sugar from malted cereal grains (e.g. barley, wheat). The production of beer consists of several stages: the transformation of barley water extract to malt (malting), the conversion of malt to wort (mashing), yeast pitching, fermentation of sugars to ethanol and post-fermentation operations. The main post-fermentation operations are beer clarification/filtration, packaging and pasteurization. The hops added during production are responsible for the bitter flavour and contribute to its natural preservation. The beer ingredients (e.g. water, cereal, hops, and yeast) can be combined in different ways to create different styles of beers such as ale, lager, stout, pilsner, etc. A few regions such as Senne Valley in Belgium still use wild yeasts for spontaneous fermentation. Ale and lager are the two major classes of beers, obtained with top and bottom fermentation yeasts, respectively. An ale beer ferments with top-cropping *Saccharomyces cerevisiae* at temperatures around 15 to 20 °C. A lager beer is fermented by bottom-cropping yeasts such as *Saccharomyces carlsbergensis* (*pastorianus*) or *Saccharomyces uvarum* at temperatures ranging between 8 and 13 °C (Hardwick et al., 1995; Hornsey, 2003). Brewer's yeast has been the focus of several studies (Hammond, 1993; Linko et al., 1998; Priest and Yeasts, 2006; Stewart and Russell, 1986). Dengis and Rouxhet (1997) studied the surface properties of top- and bottom-fermenting yeast and Fleet (1998) reviewed the microbiology of alcoholic beverages.

The production of industrial bottled beer ends with a process of thermal pasteurization. This thermal process aims to inactivate the fermenting yeast used as starter along with potential spoilage microorganisms such as wild yeasts, *Lactobacillus*, *Pediococcus*, *Leuconostoc* and other bacteria that can contaminate the beer during the fermentation (Priest, 2003; Priest and Yeasts, 2006). The pasteurization enables the stabilization of the beverage for a longer period, increasing the beer shelf-life. The pasteurization measure for beer is PU (pasteurization unit). One PU is equivalent to 1 min at 60 °C, with z = 7 °C for vegetative yeast in beer and 15 PU is the minimum pasteurization required (Carl, 1995). King et al. (1978) found that flash pasteurization at 71 °C for 30 s did not fully inactivate the beer spoilage organisms such as *Lactobacillus brevis*, *Pediococcus cerevisiae*, and a wild yeast *Saccharomyces diastaticus*. Normally, bottled beer is processed at 65–68 °C for 20 min or 72–75 °C for 1–4 min, equivalent to 10–20 PU (Portno, 1968; Fricker, 1984), since beer is carbonated, contains ethanol, has a low pH from 3 to 4.2 (Horn et al., 1997) and is bittered with hops, which are all natural antimicrobials. Therefore, thermal pasteurization is effective for its stabilization at room temperature (Silva and Gibbs, 2009). However, concerns have been expressed, especially in ethanol-free and in less bitter beers, the last being a trend in consumer preference. L'Anthoen and Ingledew (1996) reported that the D-value of lactic acid bacteria was four- to seven-fold higher in ethanol-free beer compared to 5% (v/v) ethanol beer. In addition, pathogens such as *Escherichia coli* O:157:H7 and *Salmonella typhimurium* were also more heat resistant by three to seventeen times in alcohol-free beer.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.002
0168-1605/

Presently, the beer industry applies a more severe pasteurization process (e.g. 120 to 300 PU), to cope with on-going modifications in the traditional beer composition (Silva et al., 2014).

The thermal inactivation of microorganisms is often described by first order kinetics, with D- and z-values being the parameters estimated. Buzrul (2007) used first order kinetics for modelling *S. carlsbergensis* vegetative cell survivors in beer. D-value is the time required at a given temperature to inactivate 90% of the studied microorganisms and z-value is the temperature required for a one-log reduction in the D-value (Bigelow and Esty, 1920; Silva and Gibbs, 2009). The D- and z-values are used to define beer pasteurization times at different temperatures.

Although some researchers have determined *S. cerevisiae* thermal resistance parameters, only one performed tests in beer and these experiments were carried out with vegetative cells (Tsang and Ingledew, 1982). Past work with *S. cerevisiae* in fruit juices (Put et al., 1976; Put and Jong, 1982) demonstrated that the ascospores are 25 to 350 times more heat resistant than vegetative cells, and the highest $D_{60°C}$-value for ascospores (among the 21 strains tested) was 19.2 min. Considering the huge difference between the thermal resistance of ascospore and vegetative cells, one can assume that if spores are destroyed, all the vegetative cells will also be. Lin (1979) obtained higher sporulation rates of *S. cerevisiae* when beer, barley, and malt extracts were added to the sporulation agar. This suggests it is possible to find yeast ascospores during brewing, especially due to the adverse conditions created by the ethanol, hops and carbon dioxide, all natural antimicrobial beer components. In a previous study using Pulsed Electric Fields, we have observed that the inactivation of *S. cerevisiae* ascospores was easier in high-alcohol beers (Milani et al., 2015). Hence, the study of the effect of beer alcohol content on the thermal inactivation of yeast ascospores is also important to investigate.

Therefore, the objectives of this work were to determine: (i) the sporulation rate of different brewing and non-brewing *Saccharomyces* yeast strains; (ii) the thermal resistance (D- and z-values) of ascospores of three *S. cerevisiae* strains and one *Saccharomyces pastorianus* strain in beer; (iii) the effect of beer alcohol content on the thermal resistance of *S. cerevisiae* DSMZ 1848 ascospores.

2. Material and methods

2.1. Yeast strains

The eight strains of *S. cerevisiae* and four strains of *S. pastorianus* used in this investigation were obtained from different culture collections as described in Table 1): ATCC 9080, CBS 1171 (top fermenting yeast, neo type strain isolated from beer), CBS 1503 (type strain bottom fermenting), CBS 1538 (neo type strain isolated from beer), DSMZ 1848 (hybrid isolated from bottom fermenting beer), DSMZ 70487 (isolated from super attenuated beer), Wyeast 1469 (commercial bottom fermenting brewing yeast) and Wyeast 2278 (commercial top fermenting brewing yeast). In addition the following strains from the School of Biology Sciences of the University of Auckland were used because of their good sporulation: BC186 (natural isolate from oak trees), SK1 (= NCYC 3265, lab strain isolated from soil; Liti et al., 2009), Zymaflore F15 (commercial wine yeast; Harsch and Gardner, 2013), and Lesaffre Ethanol Red® (industrial fermentation).

All the strains were tested for sporulation while for the thermal inactivation experiments the strains DSMZ 1848, DSMZ 70487, ATCC 9080, and Ethanol Red® were used.

2.2. Yeast enumeration

Colony formation was used for yeast enumeration. Yeast Extract Peptone Glucose (YEPG) medium was prepared by mixing 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 2.0% (w/v) glucose, 2.0% (w/v) agar. The agar medium was autoclaved at 121 °C for 10 min. A volume of 100 μL of appropriately diluted beer samples containing the yeast was spread into duplicate agar plates and colonies were counted after 2 days of incubation at 28 °C.

2.3. Spore production

The culture stored at -80 °C was streaked on YEPG agar and after growth a fresh single colony was inoculated into 50 mL of presporulation sterilised liquid (121 °C, 10 min) composed of 0.8% yeast extract, 0.3% peptone, 10% glucose, and zinc sulphate 25 mg/L. After inoculation, the presporulation flasks (500 mL) were incubated overnight in incubators (with rotary shaking at 168 rpm) at 28 °C. When optical density (PG Instrument T60 set at 600 nm) reached around 0.2 to 0.8, an appropriate portion of the presporulation broth (ca. 1.5 mL) was inoculated into sterile sporulation broth (10 mL) to yield 10^7 cfu/mL. Sporulation broth consisted of potassium acetate 1% (w/v), bacto yeast extract 0.1% (w/v), glucose 0.05% (w/v), zinc sulphate 25 mg/L. The mixture was incubated at 18 °C for 14 days (with rotary shaking at 230 rpm) in 1-L Erlenmeyer flasks. The solution was split in 1-mL Eppendorf tubes and the spores were extracted from the vegetative (parental cells) by adding 100 μL Zymolyase solution (5 mg/mL solid Zymolase in pH 7.2 buffer containing 1.2 M sorbitol and 0.1 M KH_2PO_4), 900 μL spheroblasting buffer (2.2 M sorbitol), and 800 μL softening buffer (100 mM Tris–SO_4, pH 9.4, 10 mM dithiothreitol (DTT) solution). Then, the mixture was incubated at 30 °C in a water bath for 2 h and the Eppendorfs were gently inverted every 20 min to accelerate the break-up of tetrads into single ascospores. The spores were harvested by centrifuging three times at 9700 *g* (rotor F-45-12-11) for 1 min and resuspending in 200 μL of 0.5% Triton X-100 to ensure total removal of the enzyme. After the last resuspension, 4 μL DTT was added to the Eppendorfs containing the spore solution. Then, the Eppendorfs were sonicated three times at 6 Hz for 2 min, both to break up tetrads into single ascospores and to kill any vegetative cells remaining in the medium. Finally, 1 mL of salt triton dithiothreitol (STD) solution (0.1 g NaCl in 10 mL of 0.05% Triton X-100) was added to the spore solution to avoid spore aggregation (Xiao, 2006).

2.4. Determination of sporulation rate

The sporulation rate was determined after 7 days of incubation and reassessed after 10 and 14 days. Strains showed different behaviours during sporulation. Some strains changed into tetrads, some triads, some dyads, and others stayed as vegetative cells. In order to measure the sporulation rate, a portion of 50 μL of the spores was diluted into 950 μL of a 1:1 mixture of sterile water and methylene blue (ca 10^7 cfu/ml) and the spores were counted under a microscope using a haemocytometer. Adding the methylene blue to the spore suspension allowed differentiating the live from dead cells, due to permeation of the methylene blue through the cell walls of dead cells. Blue-staining (dead) cells were not counted. Sporulation rate was calculated as the percentage of tetrads and/or triads divided by the total cell counts (tetrads, triads, dyads, and vegetative cells). Four replicate counts were carried out for each strain and the sporulation rate average ± standard deviation was determined. ANOVA was used to investigate significant differences between yeast strains (Statistica version 8, USA), and when differences were detected ($p < 0.05$), Tukey's Honest Significant Difference (HSD) test was carried out to separate the average values.

2.5. Saccharomyces thermal inactivation experiments

Ethanol is the major alcohol of beer fermentation by yeast. Alcohol by volume abbreviated as ABV, abv, or alc/vol is a standard measure of how much alcohol (ethanol) is contained in a given volume of an alcoholic beverage. It is expressed as a volume percent and defined as the number of millilitres of pure ethanol present in