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Development of a Pasteurization Bioindicator for Nonalcoholic Beers

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ABSTRACT

Pasteurization is still the best way to preserve nonalcoholic beers, and it is still the first choice for most brewers, but as there is still uncertainty about what level of pasteurization is required for nonalcoholic beers, finding the way to determine it is critical. This article briefly explains the key factors that may influence the heat resistance of microorganisms and, consequently, the level of pasteurization required for nonalcoholic beers and explain in detail the recently developed methodology used for the determination of the minimum pasteurization level for nonalcoholic beers, which may also be applied for other beverages. In this work ascospores of *Saccharomyces cerevisiae* BRYC501 (pasteurization bioindicator) were used to validate the minimum pasteurization required for nonalcoholic beers. Results showed that beers with low bitterness require significantly higher pasteurization units (PU) (132 PU at 5.6 IBU or 83 PU at 8.6 IBU) than beers with higher bitterness, which require as low as 30 or 34 PU for 50 and 25 IBU, respectively.

Keywords: nonalcoholic beverages, pasteurization bioindicator, pasteurization optimization

Introduction

Pasteurization has been used by the beverage industry for many decades. The main purpose of this process is the inactivation of vegetative microorganisms (bacteria and yeast) and to preserve products (1). Pasteurization doesn't target spore-forming bacteria, such as *Bacillus* or *Clostridium*, nor mold spores, because these will not be able to spoil beer due to low pH, the presence of hops, or low levels (ppb) of oxygen (2,3). The most common beer-spoilage microorganisms are lactic acid bacteria, such as *Lactobacillus* sp., *Pediococcus*, and *Leuconostoc*; anaerobic bacteria, such as *Megasphaera* and *Pectinatus* spp. (4–7); and wild yeast, such as *Saccharomyces cerevisiae* var. *diastaticus*, *Pichia*, and *Brettanomyces*, as these can ferment residual sugars that could be present in fully fermented beers (8–10).

The level of pasteurization applied to nonalcoholic beers, or any other product, is directly correlated with the heat resistance of spoilage microorganisms, which depends on the physical state of the microorganism, as well as the physiochemical properties of beer, such as pH, alcohol by volume (ABV), presence of hop components, level of sugars, carbonation, etc. (1).

It's extremely difficult to predict the physical state of beer-spoilage microorganisms at the point of pasteurization, as they could be exposed to a number of mutagens, they could gain or lose resistance following the exposure to cleaning and disinfectant chemicals used in the brewery, they could be protected (encapsulated) by various substances or not be very resistant at this particular time, and could be inactivated rapidly at pasteurization temperature. The precise process leading to cell death is complex and difficult to explain. The complexity of food and beverage matrices containing various components, both supporting and inhibiting growth, makes predictions very difficult. Even though degradation of bacterial and yeast cell walls during heat treatment is well described, the precise prediction of death in a food/beverage environment can be challenging. Microorganisms are not only exposed to heat but also to acids, bases, bacteriocins, alcohols, pH, osmotic pressure differences, oxidation reduction potential, oxidative agents, and antimicrobial agents. Furthermore, each of these factors can cause damage to the cell at different degrees and may compromise cell functions at different levels. The nature of the microbial interactions in foods/beverages or in the environment, with the matrix, or between organisms themselves and their response can determine their ability to survive. Bacterial resistance to potentially lethal treatments can be affected by the state of the cell determined by environmental conditions encountered previously. Adaptation stages can result in a decrease of the harmful effects of unfavorable conditions. Growth conditions or the exposure of microorganisms to higher temperatures can increase their heat resistance (11,12). Furthermore, preexposure of organisms to low pH also can increase their resistance to extreme acidic conditions (13). Some reactions to stress can be observed rapidly after exposure, while some responses are slower because they require gene transcription and the production of proteins, such as heat-shock proteins known as chaperones or chaperonins. Heat-shock proteins are programmed by genes (14) and are regulated by sigma factors (essential proteins in microorganisms transcriptions).

Two major stress response pathways were identified in yeast (*S. cerevisiae*) (15): they are the heat-shock response (HSR), which is activated by sublethal heat stress (16) and mediated by the heat-shock transcription factor (HSF) (17). Alternatively, the general stress response (GSR) is activated by a number of environmental stresses, including osmotic, oxidative, pH, heat, and nitrogen starvation (18–20). The GSR is thought to be an evolutionary adaptation that allows yeast to respond to adverse environmental conditions in a nonspecific way, so that cellular fecundity is retained while specific responses are activated (18,19). The GSR is typified by the upregulation of about 200 genes and their corresponding proteins, which are involved in a diverse array of cellular functions (21,22). The complexity of food and bev-

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erage matrices containing various components, both supporting and inhibiting growth, makes predictions very difficult. Even though degradation of the bacterial cell wall during heat treatment is well described, the precise prediction of bacterial death in a food environment can be particularly challenging. In beer, the main factors influencing growth, survival, or inactivation of microorganisms are alcohol, pH, bitterness, and pressure and presence of carbon dioxide, and describing their impact on microorganisms is critical.

Effect of pH

When cells are placed in a low-pH environment, undissociated lipophilic acid molecules, unlike protons and other charged molecules, can pass freely through the membrane. Once they enter the cell, the higher intracellular pH shifts the equilibrium toward the production of undissociated molecules in the cytoplasm. The cell tries to maintain its internal pH by neutralizing or exporting the protons released by the dissociation of the acid, but this inhibits growth, because energy is diverted from growth-related functions. If the external pH is sufficiently low, the burden on the cell becomes high, and the cytoplasmic pH drops to a level where damage to cellular structures, such as proteins, etc., occurs and growth is no longer possible, resulting in cell death. Adaptation of bacteria to unfavorable environmental conditions is essential for survival in acidic foods or inside the host stomach, where during gastric passage the foodborne pathogens survive a combination of inorganic and organic acids and pH values as low as 1.5–2.5 (11,23,24).

Antimicrobial Components

Antimicrobial agents are produced by a wide range of microorganisms, plants, and mammals as part of their defense against natural enemies or competitors for nutritional resources in their environment. An important role of antimicrobial compounds is to inhibit the growth of a variety of spoilage, pathogenic, and competitive bacteria. A prominent target of numerous antibacterial agents is the bacterial cell envelope, which is unique to bacteria and fulfills many crucial physiological functions. Unlike antibiotics, sanitizers are multitarget antimicrobial agents that usually act on various types of microorganisms in a concentration-dependent manner. Their mechanisms of action are rather general and involve diffusion through the bacterial cell membrane, DNA cross linkage, or elimination of spore germination (25). The nature of specific adaptive responses of microorganisms to the presence of certain antimicrobial compounds is often linked to the particular modes of action and characteristic target sites of the stress response-inducing antimicrobials; their major target sites are the bacterial cell envelope, DNA replication, and protein biosynthesis.

Hops

The impact of hop extracts on the behavior of microorganisms has been explored by many researchers (4,26–29), but there is no published data on the impact of hop extracts on the heat resistance of yeast or *Lactobacillus*. Our earlier work (30) showed that the heat resistance of beer-spoilage microorganisms was significantly lower in more bitter, German-style lagers and higher in less bitter, American-style lagers. Consequently, the calculated pasteurization units (PU) required for German-style lager (more bitter) were half those for American-style lager. In this work, we sought to validate this hypothesis and show the extent of the impact of isomerized hop extracts on heat resistance. Individual hop resin compounds are differentially effective. Although the α-acids and β-acids are generally more effective against bacteria than are iso-α-acids, in practice, their solubility in beer is significantly lower and, accordingly, their antimicrobial efficacy is less than that for iso-α-acids (31,32). Beer-spoilage bacteria, including *Lactobacillus* and *Pediococcus* spp., are resistant to the antimicrobial effects of humulone, colupulone, and transisohumulone, whereas strains that do not spoil beer are sensitive (31). Michel et al. (4) showed that different hop compounds inhibit beer-spoilage bacteria at different levels and that tetrahydro-iso-α-acids and α-acids inhibit the growth of spoilage bacteria the most. The mechanism of inhibition of sensitive bacterial cells is well understood (8,9). β-Acids, α-acids, and iso-α-acids act as mobile carrier-type ionophores and attack the plasma membranes of susceptible cells, leading to the dissipation of the trans-membrane proton gradient, which, in turn, leads to a decrease in the proton motive force, starvation, and cell death. Hop-resistant bacteria can protect themselves from hop compounds in various ways. These include a *horA* gene-dependent hop resistance mechanism and use a multidrug resistance pump (HorA) or a proton motive force-dependent transporter, as well as by pumping out protons by overexpressed H^+-ATPase following exposure to hop resins. The *horA* gene encodes an ATP-dependent multidrug transporter that removes hop bitter acids from bacterial cells.

Alcohol

The effect of alcohol on the heat resistance of microorganisms is well documented (33–36). The higher the ethanol content, the *D* values (decimal reduction time) decrease significantly, and the heat treatment is more effective. Milani et al. (33) reported a 50% reduction in yeast ascospores D_{55} value between 0.0 and 7.0% ABV beer, and Rachon et al. (30) showed that D_{60} of yeast ascospores can be over 18 times greater in nonalcoholic beer when compared with the same style of alcoholic (4.5% ABV) beer. The impact of ethanol on the heat resistance of other beer microorganisms is similar. Rachon et al. (30) showed that D_{60} of beer-spoilage bacteria is two times higher in nonalcoholic beers when compared with the alcoholic version. According to Belmans et al. (37) and Eilers and Sussman (38), chemical compounds such as ethanol were able to break the dormancy of ascospores. It was hypothesized that these compounds may act by causing an alteration in the lipid moieties of the spore. Studies on the survival of yeast vegetative cells and bacteria during exposure to ethanol have shown a clear influence of elevated temperature (30,36,39–41), which has been attributed to their combined effects on membrane composition and fluidity.

Current Developments

The EBC pasteurization guideline (42) states that the typical PU values for pasteurization of nonalcoholic beers are between 80 and 120 PU and for low-alcohol beers are between 60 and 80 PU. While included in this guidance the equation for calculation of PU can still be used, but more attention should be paid to the *z* value used. PU are known in German as pasteurization Einheit (PE) and in French as unité de pasteurization (UP). PU are used to measure the intensity of heat treatment applied during pasteurization: 1 PU corresponds to 1 min of heating at 60°C (or 140°F); 2 PU corresponds to 2 min of heating at 60°C (or 140°F); 3 PU corresponds to 3 min of heating at 60°C (or 140°F), etc. PU also can describe the intensity of pasteurization at different temperatures, which can be calculated using Equation 1, or PU of the entire process can be calculated using Equation 2.

Equation 1 is used for to calculate PU at a set temperature:

$$PU = t_T \times 10^{(T-T_{Ref})/z}$$

Equation 2 is used to calculate PU at any temperature profile:

$$PU_{tot} = \Delta t_T \times \sum_{0}^{t} 10^{(T-T_{Ref})/z}$$

PU: Pasteurization unit
PU_{tot}: Pasteurization unit (entire process)
t_T: Time at temperature T
T: Temperature
T_{Ref}: Reference temperature (60°C or 140°F)
z: z value (6.94°C)

As this guidance was published almost three decades ago, at a time when nonalcoholic beers were not as popular as today, there is simply not enough evidence for clear justification of these values. Although these values are likely effective, very often effective pasteurization can be achieved under much lower pasteurization conditions. Previous work has shown that some typical nonalcoholic beers can be pasteurized at as low as 40 PU (43), which is almost half of the recommended typical values in the EBC guidance (42). Similarly, current data (not yet published) suggest that some nonalcoholic beers (high IBU and low pH) can be pasteurized at 20 PU (four times lower than recommended values). After alcohol, pH and bitterness are two main factors contributing to the heat resistance of microorganisms and required PU. The highest pasteurization would be required at the lowest bitterness and highest pH, and vice versa, the lowest pasteurization would be required for beers with lowest pH and highest bitterness (43,44). Although this sounds obvious, it was first proved only a few years ago (43). Similarly, this was observed for other beverages when the suggested level of heat treatment was high, but they can be effectively pasteurized under much lower pasteurization conditions (Table 1).

Development of Pasteurization Bioindicator

A pasteurization bioindicator (ascospores of *S. cerevisiae* BRYC501) was established following a series of studies started in 2017. First, the heat resistance of beer-spoilage bacteria was investigated, and existing assumptions about beer pasteurization were challenged. Next, studies were focused on yeast and, more precisely, yeast ascospores (44), which showed that bitterness is one of the key factors influencing the heat resistance of microorganisms influencing required pasteurization level (43). Finally, the best practices for preparation, storage, and recovery of the ascospore were investigated and published (45). In the first article published on this work (46), it was shown that using a standard z value (z = 6.94°C, determined first by Del Vecchio) for calculation of lethality during pasteurization does not always give correct results, as z values for different microorganisms vary. For some microorganisms (*Lactobacillus brevis* BSO566), z values are higher (z = 9.48°C) than 6.94°C, and for other microorganisms (*Acetobacter pasteurisanus* BSO547), z value are lower (z = 5.17°C) than 6.94°C. In effect, microorganisms with lower z values will be more heat resistant than microorganisms with higher z values at lower temperatures and vice versa; microorganisms with higher z values will be more resistant than those with lower z values at higher temperatures (Fig. 1). To avoid using two microorganisms for pasteurization validation, it was concluded that using more heat-resistant microorganisms might be more suitable. Consequently, a second study was commenced with a focus on finding more heat-resistant microorganisms and on performing a series of heat-resistance trials of this microorganism in alcoholic and nonalcoholic beers. In this study, the yeast ascospores were identified as a potential heat-resistant microorganism that could be significantly more heat resistant than beer-spoilage bacteria and, therefore, perfect for use as a pasteurization bioindicator. After screening more than 60 yeasts, 30 of them were identified as ascospore producers, and 1 of them (*S. cerevisiae* BRYC501) produced the most heat-resistant ascospores and was selected for further studies. The heat resistance of its ascospores was tested in premium and nonalcoholic beers, and results showed that ascospores of *S. cerevisiae* BRYC 501 were significantly more (over 6–16 times) heat resistant than heat-tolerant lactic acid bacteria. The D and z values of *S. cerevisiae* BRYC 501 ascospores were determined in alcoholic and nonalcoholic versions of two lager beers (American and German styles). The spores were over 14–18 times more heat tolerant in the nonalcoholic beers and, accordingly, higher PU need to be applied.

Interestingly, at the same or similar alcohol concentration and pH, the yeast ascospores were significantly more heat resistant (1.9–2.5 times) in the American-style than in the German-style beer, which may suggest that bitterness contributed to their heat resistance. Consequently, low-bitterness beers (American-style lager) required much more pasteurization (more than twice the pasteurization) than beers with higher bitterness. The impact of bitterness on the heat resistance of both ascospores and beer-

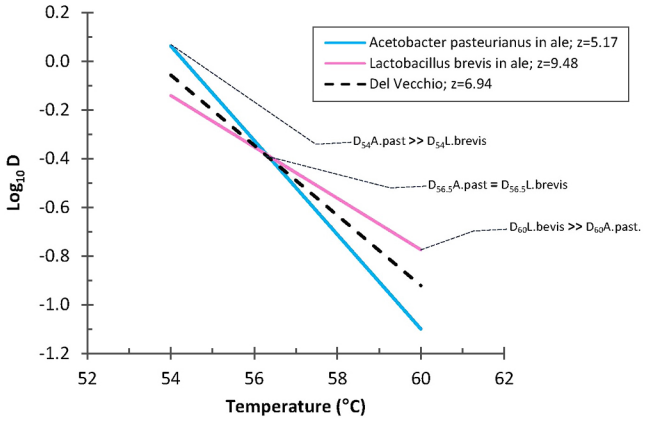

Figure 1. The importance of z values and the heat resistance switch between *Acetobacter pasteurianus* and *Lactobacillus brevis* (46).

Table 1. Typical and effective pasteurization units for different beverages[a]

Beverage	ABV (%, vol/vol)	pH Units	Bitterness (IBU)	D_{60} (min)	z Value (°C)	Effective Pasteurization Units	Typical Pasteurization Units
Lager (premium)	3.5–4.9	4.2–4.4	7–25	0.5–3	3.5–4.5	3–20	15–25
Lager (nonalcoholic)	0–0.5	4–4.5	5–50	5–25	2.3–4.2	30–140	80–120
Hard seltzer	3–5	3	N/A	0.5–3.5	3–4	3–25	N/A
Cider	4–5.5	3.2	N/A	0.1–2	4–5	0.5–12	N/A
Other low-pH nonalcoholic beverages	0	3–3.7	N/A	4–6	3.2–5	24–40	300–500

[a] ABV: alcohol by volume; N/A: not available.

spoilage bacteria was investigated further in 2022 (43), when it was confirmed that higher bitterness in nonalcoholic beers required significantly less (two times) pasteurization (41 PU) than less bitter beers (57 PU for 25 IBU or 83 PU for 8.6 IBU). This also was observed in alcoholic beers, but the impact of bitterness in nonalcoholic beers on the heat resistance and pasteurization level was much greater. Similarly, the impact of bitterness on the heat resistance of beer-spoilage bacteria was also greater in nonalcoholic beers. In addition to bitterness, pH is a key factor influencing the heat resistance of many microorganisms. The impact of pH on the heat resistance of beer-spoilage microorganisms or yeast ascospores has not been studied extensively in beer environments, but results, not yet published, show that at lower pH (pH 3.8–4.2) the heat resistance of yeast ascospores was significantly lower (D_{60} = 4.2–9.2 min), and similarly, at higher pH (pH 4.5–5) the heat resistance of yeast ascospores was much higher (D_{60} = 10.1–13.2 min). As nonalcoholic beer recipes are still evolving, the pH range of these beers is large. Results of pH and bitterness testing of 50 nonalcoholic beers available on the U.K. market showed that the pH of these beers can be in the range of 3.8 to 4.8, and the measured bitterness of these beers is in the range of 8 to 45 IBU (47). This indicated that in some cases (low-bitterness and high-pH) beers may not only require a substantial level of pasteurization but also can support the growth of other microorganisms that are not normally regarded as beer-spoilage microorganisms, such as *Bacillus cereus*, or pathogenic microorganisms, such as *Escherichia coli* or *Salmonella*.

Establishing the correct pasteurization level is critical, as it will give brewers confidence that the product will be pasteurized correctly and shelf stable after pasteurization but not over pasteurized, so energy, time, and other resources are not wasted. (Not to mention the impact on beer quality.) In most situations, the pasteurization levels are estimated based on the available data (D and z values), and pasteurization occasionally is validated with spoilage microorganisms but not always in the worst-case scenario. Although the heat resistance of key beer-spoilage bacteria and yeast has been investigated by many scientists over the past few decades, some results have been inconclusive due to the use of different methodologies. Some report that the heat resistance of beer-related microorganisms was very low, D_{60} = 0.45–2.6 min in nonalcoholic beer (44,48), while some scientists report that the heat resistance of these microorganisms was significantly greater. According to L'Anthoën and Ingledew (34), the D_{60} of *Pediococcus acidilactici* ATCC8042 in nonalcoholic beer was D_{60} = 7.6 min, with a z value of 49.3°C, and D_{60} = 1.33 min, with a z value of 24.6°C (34). Using these microorganisms for calculation of the minimum pasteurization level may not be very precise, as it is unknown how these microorganisms will behave in different beers at different pHs and different IBU. Although pasteurization can be calculated from many other D and z values (Table 2), these values will be different in different beers; therefore, heat resistance of the most heat-resistant microorganisms should be established in specific beers. As mentioned earlier, the microorganisms that characterize the highest resistance are ascospores of *S. cerevisiae* BRYC501, and they should be used for pasteurization validation of nonalcoholic beers, as well as for other non-, low-, and alcoholic beverages, where typical spoilage microorganisms are identified as yeast and spoilage bacteria. The use of this ascospore as a bioindicator should assure that PU calculations are conservative and that pasteurization schedules for low bitterness and nonalcoholic beers are effective.

Materials and Methods

Optimization of pasteurization of nonalcoholic beers is established in two phases. In the first phase, D and z values of the most heat-resistant microorganism (ascospores of *S. cerevisiae* BRYC501) that are able to spoil beer are experimentally determined. In the second phase, the minimum pasteurization level required for inactivation of at least 6 log of this microorganism is calculated, and then, the real lethality of the optimized process is verified in a tunnel pasteurizer in the brewery or pilot plant.

There are many different methods for the determination of D and z values, but the most common is using glass capillary tubes. The main advantage of this method is the rapid heat transfer of thin glass (capillary tubes), so the target temperature inside the capillary tube is achieved within seconds of starting the heat-inactivation experiment. The heat-inactivation experiments normally are determined at a range of temperatures. Selected temperatures should cover the range of working temperatures for pasteurization, as extrapolation of D values outside the tested temperatures may result in inaccurate values. In practice, heat resistance is determined at four or five temperatures, so the z values are calculated with a high coefficient of determination (R^2). For nonalcoholic beers pasteurized at around 60°C, the recommended temperatures are 60°C but also a couple of temperatures below this temperature (56 and 58°C) and a couple of temperatures above this temperature (62 and 64°C). Of course, this may not always be possible, especially if tested microorganisms are very heat sensitive in tested products and are rapidly inactivated at tested temperature or vice versa if tested microorganisms are extremely resistant in tested products. As the ascospores of *S. cerevisiae* BRYC 501 were identified as the most heat-resistant microorganism that could spoil beer, this microorganism is used for heat-inactivation experiments. The ascospores of this microorganism are produced on ascospore agar (AA) (HiMedia, India). AA is inoculated with a 48-h culture of yeast in yeast and mold (YM) broth (Oxoid, UK), and after 10 days of incubation at 25°C, the ascospores are harvested using L-shaped spreaders and SDW (sterile distilled water). The ascospore solution is then centrifuged down using a microcentrifuge (3,000 × g for 5 min). The supernatant is discarded, and the pellet is suspended in each sample. The D and z values of the yeast ascospores are then determined in the product using the capillary tube method. Briefly, 50 µL of the test solution containing between 10^7 and 10^8 spores/mL is introduced into soda-glass capillary tubes, and the tube ends are heat sealed with a flame and processed within 15 min. The sealed capillary tubes are submerged in a water bath set at the required temperature for the tested product and held for a specific length of time. For each trial, the inactivation of ascospores is monitored at five to nine holding times. Following the heat treatment, the tubes are promptly removed from the water bath and cooled down in ice water, the liquid is recovered in Ringer's solution, and the number of viable cells is enumerated by spread plating. The live yeast ascospores are recovered on YEPG agar (yeast extract: 5 g/L; glucose: 20 g/L; peptone: 10 g/L; agar: 20 g/L) and counted after 10 days of aerobic incubation at 27 ± 1°C (45). The D and z values are then calculated using Minitab software. Each heat-inactivation experiment should show at least 3 log reductions of initial inoculum, with desirable 5 or 6 log reductions this should be obtained from at least five time points. Once D and z values are determined and the linear inactivation is observed ($R^2 \geq 0.95$) for both D and z values, this data can be used

Table 2. *D* and *z* values of microorganisms related to nonalcoholic beers[a]

Microorganism	Beer Details	Temperature (°C)	*D* Value (min)	*z* Value (°C)	Reference
Bacteria					
Lactobacillus–E93	<0.05% lager, pH 4.2	60	2.6	12.1	48
Escherichia coli O157:H7–ATCC 43889	≤0.5% lager, pH 5.2	60	0.3[b]	7.3	34
Lactobacillus delbruecki	≤0.5% lager, pH 5.2	60	4.01[c]	15.5	34
Pediococcus acidilactici–ATCC 8042	≤0.5% lager, pH 5.2	60	7.7[d]	49.3	34
Salmonella typhimurium–ATCC 14028	≤0.5% lager, pH 5.2	60	0.04[e]	4.4	34
Lactobacillus frigidus Stamm-Nr. L 150	Alcohol-free	50	3.3	27.9[f]	55
Lactobacillus lindneri Stamm-Nr. L 2	Alcohol-free	59	0.7	54.8[f]	55
Lactobacillus lindneri Stamm-Nr. L 2	Alcohol-free	59	10.5	11.2[f]	55
Lactobacillus brevis–BSO 566	0% lager, pH 4.4, 25.9 IBU	60	0.7	N/A	44
Lactobacillus brevis–BSO 566	0% lager, pH 4.4, 5.6 IBU	60	1.3	N/A	44
Lactobacillus lindneri–BSO 943	0% lager, pH 4.4, 25.9 IBU	60	0.5	N/A	44
Lactobacillus lindneri–BSO 943	0% lager, pH 4.4, 5.6 IBU	60	0.6	N/A	44
Mold					
Aspergillus niger–ATCC 16404	Alcohol-free	55	8.9	10.6[f]	55
Yeast (vegetative cells)					
Saccharomyces spp. XY66	<0.05% alcohol-free	60	0.5	5.5	56
Pichia anomala Stamm-Nr. P 27	Alcohol-free	58	0.4	12.3[f]	55
Saccharomyces cerevisiae var. *diastaticus* Stamm-Nr. S 22	Alcohol-free	62	1.3	7.4[f]	55
Yeast (ascospores)					
Saccharomyces cerevisiae–BRYC501 (ATCC 9080)	0% lager, pH 4.4, 8.6 IBU	60	12.5	3.6	43
Saccharomyces cerevisiae–BRYC501 (ATCC 9080)	0% lager, pH 4.4, 25 IBU	60	8.6	4.0	43
Saccharomyces cerevisiae–BRYC501 (ATCC 9080)	0% lager, pH 4.4, 50 IBU	60	5.9	4.2	43
Saccharomyces cerevisiae–BRYC501 (ATCC 9080)	0% lager, pH 4.4, 25.9 IBU	60	8.7	2.8	44
Saccharomyces cerevisiae–BRYC501 (ATCC 9080)	0% lager, pH 4.4, 5.6 IBU	60	21.9	2.3	44
Saccharomyces cerevisiae var. *diastaticus*	Heineken 0.0	52	4.1	N/A	57
Saccharomyces cerevisiae var. *diastaticus*–AB183	<0.05% alcohol-free	60	7.7	3.9	56
Saccharomyces spp.–XY66	<0.05% alcohol-free	60	23	4.1	56
Saccharomyces cerevisiae–DSMZ 1848	0% ABV	50	61.2	N/A	33
Saccharomyces cerevisiae–DSMZ 1848	0% ABV	55	34.2	N/A	33

[a] N/A: Not available; ABV: alcohol by volume.
[b] D_{60} extrapolated from *z* value of 7.3°C calculated from D_{49}–D_{55} min.
[c] D_{60} extrapolated from *z* value of 24.6°C calculated from D_{47}–D_{53} min.
[d] D_{60} extrapolated from *z* value of 49.3°C calculated from D_{49}–D_{55} min.
[e] D_{60} extrapolated from *z* value of 4.4°C calculated from D_{47}–D_{53} min.
[f] *z* value data obtained from two time points.

for calculation of minimum PU. This can be calculated by simply multiplying the D_{60} value by 6 if the pasteurization will be performed at 60°C or by calculating lethality for a specific temperature profile gathered from a typical pasteurization run.

Pasteurization Verification Trials

Verification trials are performed to confirm that the PU calculated from experimentally determined *D* and *z* values are valid when the product is pasteurized in the pasteurizer in its original packaging (bottles or cans). Ideally, these trials can be performed in the brewery's pasteurizer or in a smaller scale pasteurizer that can mimic the pasteurization temperature profile in the pilot plant. It is best to perform a number of trials and test as many samples as possible, but the minimum number of replicates is three. For each trial, samples are inoculated with approximately 10^6 ascospores/100 mL and approximately 10^8 CFU of vegetative beer-spoilage bacteria per 100 mL: *Lactobacillus brevis* BSO 566 (46) or *Enterococcus faecium* NRRL B-2354, which is commonly used in the food industry as a pathogenic surrogate for validation of various thermal processes performed at different temperatures (60–140°C) (49–52), depending on the product tested. Immediately after inoculation, the bottles or cans are closed, mixed, and the number of inoculated ascospores and bacteria are enumerated by spread plating. Yeast are enumerated on YEPG agar, *Lac-*

tobacillus brevis BSO566 is enumerated on de Man, Rogosa and Sharpe agar (Oxoid), and *Enterococcus faecium* NRRL B-2354 on tryptone soya agar (Oxoid). Yeast colonies are counted after 10 days of aerobic incubation at 27 ± 1°C, *Lactobacillus brevis* BSO566 colonies are counted after 7 days of anaerobic incubation at 27 ± 1°C, and *Enterococcus faecium* NRRL B-2354 are counted after 48 h of aerobic incubation at 37°C. In parallel, 3, 5, or 10 inoculated replicates of bottles or cans are placed in the pasteurizer, and the pasteurization process is started. Following pasteurization, the number of surviving ascospores and vegetative bacteria are enumerated by membrane filtration, and the number of surviving spores and cells in 100 mL is determined. For each pasteurization trial, the temperature inside the pasteurizer and the core temperature of the tested bottles or cans (dummy bottles) is measured and logged by a calibrated temperature logger, such as WiFi-TP–Temperature Data Logger (Corintech Ltd, UK). Successful validation will result in achieving over 6 log reduction of yeast ascospores and full inactivation of beer-spoilage microorganism *Lactobacillus brevis* BSO566 and pathogenic surrogate *Enterococcus faecium* NRRL B-2354.

Results and Discussion

Results achieved by this method allow brewers to adjust pasteurization requirements for many different nonalcoholic beers. The *D* and *z* values obtained by this method for nonalcoholic beers and calculated PU are shown in Table 3. As shown, the minimum PU are between 30 and 130. In low-bitterness beers, the heat resistance of the pasteurization bioindicator was significantly higher D_{60} = 21.9 min and *z* = 2.3°C in 5.6-IBU beer. In higher bitterness beers, these values were significantly lower: D_{60} = 4.9 min and *z* = 3.8°C in 50-IBU beer and D_{60} = 5.6 min and z = 3.8°C in 25-IBU beer. Consequently, the recommended minimum pasteurization values for these beers were 132, 30, and 34 PU, respectively. Although a value of 132 seems to be extremely high for beer pasteurization (and is 12 PU outside of the typical PU values recommended by the EBC [42]), the impact of very low bitterness on the heat resistance of the bioindicator and any other beer-spoilage microorganisms is very low.

In fact, if this product was not considered beer, due to its extremely low bitterness the pasteurization recommendation for non-beer beverages would be to pasteurize at 70°C for 5 min (equivalent to 138 PU) at pH <3.5; at 85°C for 5 min (equivalent to 20,000 PU) at pH = 3.5–4.0; or at 95°C for 5 min (equivalent to 550,000 PU) at pH 4.0–4.2 (53,54). For more typical beers, those with bitterness of 10 IBU and higher, the recommended PU for tested beers are lower than those recommended by the EBC (42).

Table 3. The minimum pasteurization units (PU) obtained after validation trials with *Saccharomyces cerevisiae* ascospores BRYC501 recommended for tested nonalcoholic beers, including *D* and *z* values at different pH and bitterness levels

pH	Bitterness (IBU)	D_{60} (min)	*z* Value (°C)	Minimum PU
4.4	5.6	21.9	2.3	132
4.4	8.6	12.5	3.6	83
4.5	6	11.0	3.4	66
4.5	10	10.1	3.4	61
4.4	25	8.6	4.0	57
4.2	6	9.2	3.5	55
4.2	26	8.7	2.8	52
4.5	25	8.3	4.1	50
4.2	10	8.1	3.6	49
4.2	50	7.0	4.0	42
4.4	50	5.9	4.2	41
4.2	25	6.3	3.8	38
4.2	25	5.6	3.8	34
4.5	50	4.9	3.8	30

The methodology presented here is suggested for use on nonalcoholic, as well as on low-alcohol, standard (alcoholic) beers and other low pH beverages that would not support the growth of heat-resistant molds or spore-forming bacteria, such as *Alicyclobacillus*, *Clostridium*, or *Bacillus* spp.

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